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THESIS – THÈSE

Title of Thesis – Titre de la thèse

Ureaplasma urealyticum urease : Enzymatic and Immunochemical characterization

Degree for which thesis was presented – Grade pour lequel cette thèse fut présentée	Year this degree conferred – Année d'obtention de ce grade
M. Sc	1985
University – Université	**Name of Supervisor – Nom du directeur de thèse**
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Ureaplasma urealyticum Urease: Enzymatic and Immunochemical Characterization

by

Hubert Eng

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

Department of Microbiology

EDMONTON, ALBERTA

SPRING 1985

THE UNIVERSITY OF ALBERTA

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ABSTRACT

Ureaplasma urealyticum, an agent of non-gonococcal urethritis and other maladies, is distinguished from other mycoplasmas by the presence of the enzyme urease. Kinetic parameters of four ureaplasma strains were examined; the Km was found to be 2.5 mM urea, and the pH optimum 7.2. The value for the pH optimum is greater than stated in previous reports; no explanation has yet been found for this discrepancy. Ureaplasma urease was found to have a molecular weight of 380,000 by native gel electrophoresis, and 330,000 by molecular sieving. Using isoelectrofocusing, most of the ureaplasma strains possessed 2 to 3 enzyme forms at or close to a pI of 5.2.

Rabbit antiserum against ureaplasma strain 8 urease was generated and used to determine the extent of neutralization of urease from other ureaplasmas and from several Gram positive and Gram negative eubacteria. Neutralization levels varied from 17% for the homologous antigen, to 73% for the bovine isolate. Urease from the eubacteria was not inhibited by the antiserum. To explain the low level of inhibition for homologous urease and the high levels of inhibition for heterologous urease, a model was proposed in which ureaplasma urease possessed two epitopes, I (inhibiting) which was invariant and responsible for neutralization and P (protecting) which was variable and determined the extent of neutralization.

ACKNOWLEDGEMENTS

I would like to thank Dr. G.W. Stemke for his guidance, advice, and patience that enabled me to complete this work. I would also like to thank Dr. J. Robertson and all those on my committee whose contributions to the research were invaluable.

I would like to acknowledge the financial assistance of the Alberta Heritage Foundation for Medical Research which made the research possible (Studentship AHFMR RA1629).

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I. Introduction

A. Overview of the Class *Mollicutes*

Mollicutes lack a cell wall, are bound by a trilaminar membrane, and are the smallest free living self replicating organisms. Although many species of *Mollicutes* have a unique morphology, *Mollicutes* as a whole may be regarded as pleomorphic (69, 36). *Mollicutes* are prokaryotes with circular, double - stranded DNA, a characteristically small genome (70), and a low guanine plus cytosine content (ranging from 23 to 41 mol%, with most having approximately 33 mol%) (58, 70). There is only one order, *Mycoplasmatales*, which is divided into 3 families: *Mycoplasmataceae* (with two genera, *Mycoplasma* and *Ureaplasma*), *Acholeplasmataceae* (with one genus *Acholeplasma*), and *Spiroplasmataceae* (with one genus *Spiroplasma*). Of the *Mycoplasmatales*, only *Acholeplasma* does not require sterol. *Mycoplasma* and *Ureaplasma* have an approximate genome size of 500 megadaltons, while that of *Acholeplasma* and *Spiroplasma* is 1000 megadaltons (28). In this thesis, the general term "mycoplasmas" will be used to denote any species in the class *Mollicutes*, and the term "ureaplasmas" shall refer to members of the genus *Ureaplasma*.

B. Evolution of Mycoplasmas

The small genome of mycoplasmas led investigators to postulate two evolutionary models. The first suggested mycoplasmas were primordial organisms from which eubacteria evolved (62, 121). The second model proposed that mycoplasmas evolved from eubacteria which had lost much of their DNA including that which determined their capacity to synthesize cell wall precursers. According to this model mycoplasmas originated from either a small number of eubacteria that degenerated and diversified, or from numerous eubacteria that each underwent degenerative evolution.

Accumulating evidence suggests mycoplasmas descended from eubacteria. Studies by Neimark (64), and Neimark and London (65) have shown acholeplasmas to be related to streptococci based on RNA-DNA hybridization studies and immunological cross reactivity of various enzymes. Based on comparisons of 16S rRNA (124), 5S rRNA, and tRNA (120) oligonucleotide catalogs, as well as lipid composition (34), it has been suggested that *Mycoplasma*, *Acholeplasma* and *Spiroplasma* arose from Gram positive eubacteria, more specifically from the *Bacillus*, *Lactobacillus* lineage (and two *Clostridium* species, *C. ramosum*, and *C. innocuum* considered to be atypical clostridia (43) based on rRNA homologies).

C. *Ureaplasma urealyticum*

Ureaplasma urealyticum was first isolated by Shepard (92) from urethral swabs of patients with or without non-gonococcal urethritis and assigned the temporary designation "T," or "tiny," strain mycoplasma because of the minute colonies (7-15 μm in diameter (92)). The major feature that distinguishes ureaplasmas from other mycoplasmas is the presence of urease (93). This unique property lead to the establishment of a new genus in the family *Mycoplasmataceae* with the official binomial *Ureaplasma urealyticum* (a "urea requiring, urea hydrolyzing" organism) (99). The initial difficulty in isolation and characterization of this new found organism was due to inappropriate and inadequate growth conditions. Not until it was realized that ureaplasmas preferred an enriched and slightly acidic growth medium (97, 94) did growth yields and colony size substantially increase.

Of the human genital isolates there are 8 (99) to 14 (80) antigenically distinct serotypes, with serovar 8 being the type strain, ATCC T960. Many of the strains exhibit some degree of serological cross reactivity with each other (67, 80, 117). Though the guanine plus cytosine content of the first eight serovars are similar (1, 6), hybridization experiments with DNA (10) and comparison of polypeptides by polyacrylamide gel electrophoresis (41) reveal two clusters consisting of strains 1, 3, and 6, and of strains 2, 4, 5, 7, and 8.

Ureaplasmas were also isolated from cattle, monkeys, cats, and swine (113), sheep and goats (53), dogs(17), turkeys (105), and chickens (104). The best characterized animal ureaplasmas are those isolated from cattle. Howard and Gourlay (38) have found bovine isolates to be distinct from human isolates (based on guanine plus cytosine content, polypeptide differences using SDS polyacrylamide gel electrophoresis, and serological studies) and have suggested they be regarded as separate species, *Ureaplasma diversum*. It is uncertain whether other animal ureaplasmas which have been reported to be serologically distinct from human isolates (42, 47) can also be regarded as one or more separate species.

D. Significance of Ureaplasmas

In humans, ureaplasmas are one of the etiologic agents of nongonococcal urethritis (NGU) (112, 9), and are strongly correlated with production of chorioamnionitis (inflammation of the placenta, fetal membranes, and umbilical cord) (100), low birth weight (without decreased gestational age) (8), and arthritis (in agammaglobulinaemic patients) (108, 122). Whether ureaplasmas are causative agents of reproductive failure (e.g. spontaneous abortions, stillbirths) is still controversial, as studies both supporting (107, 82, 49, 68) or denying (61, 63, 72, 106, 118) any correlation between ureaplasma isolation and these disease states have been reported. Treatment of ureaplasma infections with

tetracycline or tetracycline analogues has been complicated by presence of tetracycline resistant strains (27, 77, 85, 101). Pathogenicity of animal ureaplasmas varies with the host as they are reported to cause pneumonia (40), conjunctivitis (35), and vulvitis (89) in cattle, infertility in sheep (54), porcine (102), and turkeys (103), as well as various disorders in other animals (16, 2, 50).

Alkaline urine, the crystallization of struvite ($MgNH_4PO_4.6H_2O$) and eventual formation of urinary stones, is associated with certain urease-producing bacteria (87, 91). Whether ureaplasmas induce, or participate in, formation of urinary stones is inconclusive. Friedlander and Braude (30) found both viable and heat killed ureaplasmas were equally capable of inducing stone formation, leading them to believe ureaplasma cells acted as a nidus for stone formation and urease activity was not essential (30). More recent studies (66, 11) however, indicate that stone formation is concomitant with ureaplasma urease activity.

Ureaplasmas are the only mycoplasma species thus far examined with an immunoglobulin A (IgA) protease (46, 81). As ureaplasmas colonize mucosal membranes, an IgA protease could reduce the effects of an immune response and enhance pathogenicity. The protease is extracellular (46), specific for IgA1 but not IgA2, IgM, or IgG (81, 46), and present in all 14 serotypes (81).

E. Morphology of Ureaplasmas

In fluid culture ureaplasmas are round to ovoid (0.3 to 1 μm in diameter) (24, 94, 123), though under suboptimal conditions they have been described as short rod-shaped (116) or partly branched filamentous cells (5). A gelatinous matrix possibly composed of carbohydrates surrounds the cell (123, 79). Although little work has been done on ureaplasma replication, it may involve binary fission similar to other mycoplasmas (78).

Ureaplasma colonies vary in size from 20 (99, 114, 115), to 120 μm (76) in diameter, with variation probably due to growth conditions. Under optimal conditions some ureaplasma strains form what has been described by Razin and Oliver (71) as a characteristic fried egg shape colony, due to surface growth at the periphery and downward growth in the center.

F. Culture Characteristics

Optimal growth of ureaplasmas occurs at pH 6.0 (96), with viability rapidly declining at pH values greater than 8.0 (97). Both serum (10 to 20%) and urea (0.25 to 1% w/v) are essential supplements (94, 75, 99). Urea concentrations greater than 1% are inhibitory (97), probably due to rapid accumulation of ammonia which becomes toxic at 200 μg/ml (25). Growth occurs slowly at 4°C (33, 22), is optimal at 35-37°C (32, 98), and absent at 42 or 45°C (3, 33). Several different media for growth of ureaplasmas have been reported

(39, 3, 75), with the maximum cell density reaching 10^7-10^8/ ml after 16 to 20 hours (75).

Although ureaplasmas have been described as microaerophilic (99), Robertson (76) has shown that many laboratory adapted strains grow nearly as well in air as in the presence of added carbon dioxide. To ensure isolation of clinical specimens, however, 95% N_2 - 5% CO_2 affords the most reliable atmospheric conditions (76).

G. Urease

Urease is present in many bacteria (86), plants, molds and yeasts (90). Established purification protocols (109, 18) have permitted extensive biochemical characterization of jack bean urease, the first enzyme to be crystallized (109). The molecular weight of native urease (α-urease) has been reported by Dixon et al. (14) to be 590,000 (±30,000) by equilibrium ultracentrifugation. As many as 12 electrophoretically different forms have been observed (19) due to aggregation or dissociation of α-urease. Aggregation may be due to air oxidation of numerous sulfhydryl groups present near the surface of the enzyme (37, 74). α-Urease is composed of two ionically linked subunits (termed A1) with each A1 subunit containing three 97,000 dalton protomers (20, 14). Jack bean urease is a unique metalloenzyme possessing two nickel ions in each 97,000 dalton unit (13) which contribute to the catalyic reaction by activating the substrate towards "nucleophilic attack on carbon by virtue

of O-coordination to an active-site Ni(II)" (7, 15). The kinetic properties of jack bean urease (that is the pH optimum, Km and so forth) vary with each preparation and may reflect "temperature dependent interconversions of two or more types of active sites (directly or indirectly through enzyme conformational changes)" (55). Hence reported pH optimum values range from 7.0 to 9.0, and Km values range from 2.7 to 4 mM urea (73).

As bacterial ureases have not been purified, extensive analysis has not been accomplished. Reported molecular weights of bacterial urease vary from 800,000 for *Proteus vulgaris* to 230,000 for *Klebsiella aerogenes* (87, 111, 31). The pH optimum also varies with the species, ranging from 6.5 to 7.5 (44, 56, 52, 51), and the Km ranges from 0.7 to 40 mM urea (31, 52, 51, 57).

H. Ureaplasma Urease

Urease of *Ureaplasma urealyticum* is reported to be constitutive, intracellular (59, 60) and not membrane bound (59, 119). Urease activity is critical for growth as substrate analogs such as the hydroxamic acids (sorbyl-, benzoyl-, 3-amino-benzyl-, and acetohydroxamic acid) (23, 60), and fluorofamide (45) are initially bacteriostatic, then bacteriocidal. Urease is also inhibited by heavy metals and divalent cations (84), but not by either product of urea hydrolysis (84).

Upon cell lysis, urease activity is reported to be relatively stable for 5 weeks if stored at -70°C; however all activity is lost within 3 weeks at -20°C (60), and 50% loss of activity occurs within 10 minutes at 37°C (84). Activity rapidly declines at pH values above 8.0 (110). Optimal enzymatic activity was detected at pH 5.0 to 6.0 (96, 60), and at urea concentrations of 5.6 mM to 20 mM urea for strain 8 (60, 110). The Km ranges from 2.17 to 5.0 mM urea (60, 97, 84).

Delisle (12) in 1977, and Romano et al. (84) in 1979, have suggested the multiple bands of urease activity observed after electrophoretic separation of the cell lysate represent isoenzymes. Without presenting results, Kenny (45), using SDS polyacrylamide gel electrophoresis, reported active urease to be 140,000 daltons, which upon boiling, dissociated to an inactive 70,000 dalton subunit.

As endproducts of urea hydrolysis by urease are liberated from the cell (25, 26), the metabolic significance of urease to ureaplasmas is uncertain. It has been suggested (59, 83) that urease may be involved in synthesis of ATP by a chemiosmotic mechanism, as ATP synthesis is dependent upon an active ATPase, urease, and the presence of urea (83). As yet, no other function has been ascribed to ureaplasma urease.

I. Research Objectives

Ureaplasma urealyticum is a major etiologic agent in sexually transmitted diseases, making characterization of this organism increasingly important. The small genome and low guanine plus cytosine content (27-30 mol%) of ureaplasmas approach the biological limit (25 mol%) required for functional gene products. Although the biological role of urease has not been unraveled, with such a limited genome, synthesis of unnecessary proteins would not be expected. By studying ureaplasma urease, it is hoped that the data will contribute to the elucidation of its possible pathological role, its biological role within the cell, and permit the refinement of urease specific chemotherapeutic agents for the control of ureaplasma infections.

Characterization of ureaplasma urease will be examined in two chapters. The first chapter discusses basic kinetic data, attempts at purification, the molecular weight, the presence of subunits, and isoenzymes of ureaplasma urease. The final chapter utilizes antiserum against strain 8 urease to examine cross reactivity of urease from other ureaplasmas and other bacteria. The antiserum was also used in the immunoblot procedure to further probe the biochemical characteristics.

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II. Characterization of Ureaplasma Urease

A. Introduction

This chapter shall examine the pH optimum, the effects of various assay conditions, and the Km for ureaplasma urease. While urease represents a relatively major component in ureaplasmas, it has not been purified to homogeneity. Pure urease would permit definitive kinetic and biophysical characterization. Unfortunately purification of ureaplasma urease is difficult as the amount of ureaplasma cell mass one can collect is minute, and contaminated with medium components.

Electrophoretic separation in native polyacrylamide gels was used to determine the molecular weight of the intact enzyme, and study the effects of sulfhydryl reducing agents. Pure urease has not been obtained by us or others, hence whole cell lysates were subjected to polyacrylamide gel electrophoresis, and the Fishbein stain (15) was utilized to specifically locate urease among the cellular constituents. The stain involves reduction of a tetrazolium dye by dithiothreitol to an insoluble formazan dye in localized regions of high pH due to the hydrolysis of urea by urease to ammonia and carbon dioxide. To maximize the sensitivity of the stain, the enzyme was electroblotted, or western transferred, from the gel to nitrocellulose.

Using non-denaturing, non-reducing polyacrylamide tube gels, Delisle (8) reported two to three bands of ureaplasma

urease depending on the isolate examined. Romano et al. (36) also reported three bands of urease activity from a partially purified ureaplasma urease preparation involving preliminary acetone treatment. To further explore the possibility of isoenzymes, the techniques of non-denaturing, non-reducing gel electrophoresis and isoelectric focusing were used.

B. Material and Methods

Materials

All reagents unless indicated were of reagent grade quality. Sodium hypochlorite (5.25% w/v) was obtained from Fisher Scientific Co., urea (electrophoresis purity) from BioRad, and para-nitroblue tetrazolium from Aldrich Chem. Co., Inc.. Acrylamide, bis-acrylamide, tetramethylethylene diamine (TEMED), ammonium persulfate, phenolnitroprusside, iodoacetamide, and dithiothreitol were all purchased from Sigma. Sepharose beads were obtained from Pharmacia. Cyanogen bromide and ethanolamine (scintillation grade) were purchased from Eastman Kodak. Sodium dichromate and formaldehyde were purchased from J.T. Baker, and silver nitrate from Engelhard Indus. (Aurora, Ont., Canada). Where required, ultrapure water was obtained using the Milli-Q reagent grade water system.

The molecular weight markers for gel electrophoresis (Sigma) consisted of the monomer and dimer of jack bean

urease (345,000 and 690,000 respectively), myosin (204,000), phosphorylase (116,000), and β-galactosidase (97,000); the molecular weights assigned to jack bean were those reported by Dixon et al. (11) and not those suggested by Sigma. Molecular weight markers for gel filtration (Pharmacia) included aldolase (158,000), catalase (232,000), ferritin (440,000) and thyroglobulin (669,000).

Ureaplasma Growth Conditions

Ureaplasma urealyticum strains 1, 3, 8, and 9 were obtained from Dr. J. Robertson (Med. Micro., Univ. of Alberta, Edm., Canada), and were the serovars for serotyping. All ureaplasma strains were cultured in Bromthymol Blue Broth (34) containing 10% horse serum and 0.025% (w/v) urea at 37°C. When the pH of the culture was approximately 6.9 the cells were harvested by centrifugation at 20,000 g for 45 minutes in a Beckman Type-19 rotor. The cell pellet was resuspended in a minimum volume of 0.15 M NaCl, and centrifuged again at 12,800 g for 5 minutes (Eppendorf microfuge 5414). Upon resuspension of the pellet in a minimum volume of 0.15 M NaCl, the cells were lysed by 3 cycles of freezing and thawing and stored at -70°C.

Assay for Urease Activity

To monitor urease activity, the modified Berthelot assay described by Wong and Shobe (43) was scaled down to a 1 ml microassay. Alkaline hypochlorite was prepared as

described by Creno et al. (6), and consisted of 0.625 M NaOH, and 21% (w/v) sodium hypochlorite. The substrate solution consisted of 25 mM urea, 5 mM EDTA, and 0.1 M phosphate buffer pH 7.2.

The assay procedure was as follows: 100 µl of cell lysate appropriately diluted in distilled water were added to 100 µl substrate solution and incubated at 37°C for 30 minutes. Then phenolnitroprusside and alkaline hypochlorite, 400 µl of each, were added to terminate the reaction. The entire mixture was incubated at 50°C for 5 minutes and indophenol was determined at an absorbance of 625 nm in a Gilford model 250 spectrophotometer. Enzyme activity is expressed as µmoles urea hydrolyzed per minute per milliliter.

Immunoaffinity Column

In an attempt to purify ureaplasma urease, rabbit anti-ureaplasma strain 8 urease antiserum (described in Chapter III) was used to prepare an immunoaffinity column (25). The antiserum was obtained by immunizing with urease/anti-urease immunoprecipitates isolated from two-dimensional immunoelectrophoretic agarose gels of strain 8 cell lysate, and rabbit antiserum against strain 8 cell lysate. Prior to coupling, both rabbit antiserum and Sepharose-4B (20 ml of packed beads) were equilibrated overnight in 0.1 M borate saline buffer (pH 8.0). The slurry of beads and buffer (equal volumes of each) was

raised to a pH of 11 with 1 N NaOH, followed by the addition of 80 mg cyanogen bromide. This mixture was kept stirring for 20 minutes and the pH maintained at 11 with 1 N NaOH. The reaction was terminated by extensively washing the activated beads with buffer. Then 12 mg of protein were added to the activated beads and the suspension was kept stirring overnight. Uncoupled protein was removed by washing with borate saline buffer and any remaining activated groups in the Sepharose beads were blocked by incubating with 50 ml of 1 M ethanolamine pH 8 for 2 hours. The beads were then washed in several volumes of phosphate buffered saline (PBS), 8 M urea / 0.05 M Tris-HCl pH 7.6, 0.1 M glycine, and finally in PBS.

SDS Polyacrylamide Gels

All polyacrylamide gels were prepared using a stock solution of acrylamide (40:0.4) containing 40% (w/v) acrylamide, and 0.4% (w/v) bisacrylamide. The TEMED and ammonium persulfate were added to a final concentration of 0.045% (v/v) and 1.6 mM respectively. Gels (1.5 mm thickness) were cast using the BioRad Protean Dual Vertical Slab gel electrophoresis equipment.

For SDS gel electrophoresis the procedure of Laemmli (24) was used. Polyacrylamide concentration of the stacking gel was 4.5%, and the resolving gel 8%. Samples were diluted with an equal volume of dissociating, non-reducing buffer (20% glycerol, 6% SDS, 10 mM Tris-HCl pH 8.0, and

bromphenol blue), then heated in a steam cone for 90 seconds. Samples were loaded onto the gel (30 µl maximum) and electrophoresis was conducted at 15 mA. Protein was electroblotted to nitrocellulose at 0.4 A for 2 hours at 4°C by the procedure described below. The nitrocellulose was then placed in 0.05 M citrate buffer (pH 6) for 30 minutes, and urease activity was detected by the Fishbein stain as outlined below.

Fishbein Stain

Fishbein stain solution (15) was prepared fresh, and consisted of 35 mM citrate buffer pH 6, 0.1 M urea, 0.05% (w/v) para-nitroblue tetrazolium chloride and 1.75 mM dithiothreitol. After equilibration of the gel or nitrocellulose with 0.05 M citrate buffer pH 6, the stain solution was added and allowed to incubate in the dark at room temperature. The reaction was stopped by addition of 10% acetic acid.

Silver Stain

All solutions used to silver stain polyacrylamide gels (29) were prepared with ultrapure water. Protein in the gel was fixed in a 10% acetic acid, 50% methanol solution for at least 2 hours after electrophoresis, followed by several 2 hour washes in 9.5% (v/v) ethanol. The gel was then soaked for 5 minutes in a solution containing 0.0034 M sodium dichromate and 0.0032 N nitric acid. After rinsing the gel

several times with water, silver nitrate, 0.012 M, was added and left for 30 minutes. The gel was again rinsed as above before adding the developer, containing 0.05 % (v/v) formaldehyde in 0.28 M sodium carbonate. Development was stopped by addition of 10% acetic acid.

Electroblot

The procedure of Towbin et al. (41) was used to electroblot, or western transfer, protein to nitrocellulose (Schleicher and Schuell, from North American Scientific Chemical Ltd.). Methanol was not incorporated into the reservoir buffer (0.025 M Tris-HCl, 0.192 M glycine pH 8.3) as it reduced enzymatic activity. All transfers were performed overnight at 40 mA with cooling unless otherwise stated.

Native Gel Electrophoresis

Gels and reservoir buffers were prepared similar to the procedure of Laemmli (24) except SDS was omitted. The cell lysates of ureaplasma strains 1, 2, 3, 5, 6 and 8 were prepared as already described.

The concentration of polyacrylamide in the resolving gels ranged from 4 to 10%. The stacking gel was 4.5% acrylamide except when using 4 or 5% acrylamide resolving gels, in which case the stacking gel was 3% acrylamide. Samples were diluted with an equal volume of sample dilution buffer (0.025 M Tris-HCl, 0.19 M glycine pH 8.4, 20% sucrose, and

bromphenol blue). A final volume of approximately 30 µl cell lysate and 20 µl combined molecular weight markers was loaded onto the gel. Electrophoresis was carried out at 20 mA per gel until the tracking dye migrated into the resolving gel, then the current was increased to 30 mA per gel. The portion of the gel containing the molecular weight markers was silver stained. The remainder of the gel, containing the cell lysate, was electroblotted to nitrocellulose, and urease activity was visualized using the Fishbein stain.

To assess the effects of sulfhydryl reducing agents under the conditions described by Edelman et al. (13), native gel electrophoresis was used. Equal volumes of cell lysate and a solution of 0.15 M Tris-HCl pH 7, 2 mM ethylenediaminetetraacetic acid (EDTA) containing either 0.01 M dithiothreitol, or 0.01 M mercaptoethanol (BDH) were combined. The mixture was left at room temperature for 2 hours. Alkylation of the treated cell lysate was achieved by adding iodoacetamide to a final concentration of 0.04 M, and incubating for 20 minutes at room temperature. A control without reducing agent was included. Samples were subjected to electrophoresis in an 8% polyacrylamide gel, electroblotted, and urease activity on the nitrocellulose was detected by the Fishbein stain.

Zone Electrophoresis

The procedure of Delisle (8) was used to detect isoenzymes. Gels, 8% polyacrylamide with 5 mM Tris-HCl/ 1.6 mM EDTA pH 8.5, were cast with one continuous acrylamide concentration throughout (that is, without a stacking gel). The 5 mM Tris-HCl/ 1.6 mM EDTA pH 8.5 buffer was used for both cathode and anode reservoir buffers. All samples were diluted with an equal volume of solution containing 10% sucrose, 0.05 M Tris-HCl, 1.6 mM EDTA, and bromphenol blue. Electrophoresis of samples (30 µl maximum) was conducted at 20 mA for one hour then at 40 mA for the remainder of the run. The protein was then electroblotted to nitrocellulose, and urease activity was detected by the Fishbein stain.

Molecular Sieving

Approximately 20 µg of strain 8 cell lysate in 0.5 ml of 0.1 M potassium phosphate/50 mM EDTA buffer were applied to a Sepharose 6B column (1.3 by 49 cm) at 4°C equilibrated with the same buffer. The flow rate was approximately 7 ml per hour, with 2.5 ml fractions being collected. Protein was determined at an absorbance of 280 nm, and urease activity was detected by the Berthelot assay.

Isoelectric Focusing

Slab gel isoelectricfocusing (IEF) was adapted from Righetti and Drysdale (33), and Page and Doran (31). Gels were cast in the BioRad Protean dual vertical slab gel

electrophoresis cell, and consisted of 8% polyacrylamide, 10% sucrose, and 3.75% pH 4 to 6 ampholine (LKB). The anode reservoir buffer consisted of 59 mM sulfuric acid, and the cathode reservoir buffer of 0.2 M NaOH. Before loading samples onto the gel, 30 µl of overlay buffer (10% sucrose, 10% pH 3.5-10 ampholine) were added to each well. Sucrose was added to all samples to give a final concentration of 25%. The protein was focused at a constant voltage of 200 V for 14 hours and then at 800 V for 1 hour (3600VH). The gel was then immersed in electroblot buffer for 5 minutes before transferring the protein to nitrocellulose, which was later examined for urease activity by the Fishbein assay.

C. Results

Urease Kinetics

Optimum urease activity was obtained when the final concentration of urea exceeded 10 mM urea (fig. 1). This corresponds to the optimum concentration of 20 mM urea reported by Swanberg et al. (40). The effects of Mn, a growth inhibitor for some ureaplasmas, on urease activity could not be assessed as it precipitated out of solution upon addition of alkaline hypochlorite making the absorbance values meaningless.

Of the four ureaplasma strains examined, the optimum pH for activity ranged from 7.2 to 7.5 (fig. 2). The experiment has been repeated several times, and each time the pH

Fig. 1. Optimum Urea Concentration. Enzymatic activity was determined as a function of final urea concentration. Each point represents a median of duplicate points.

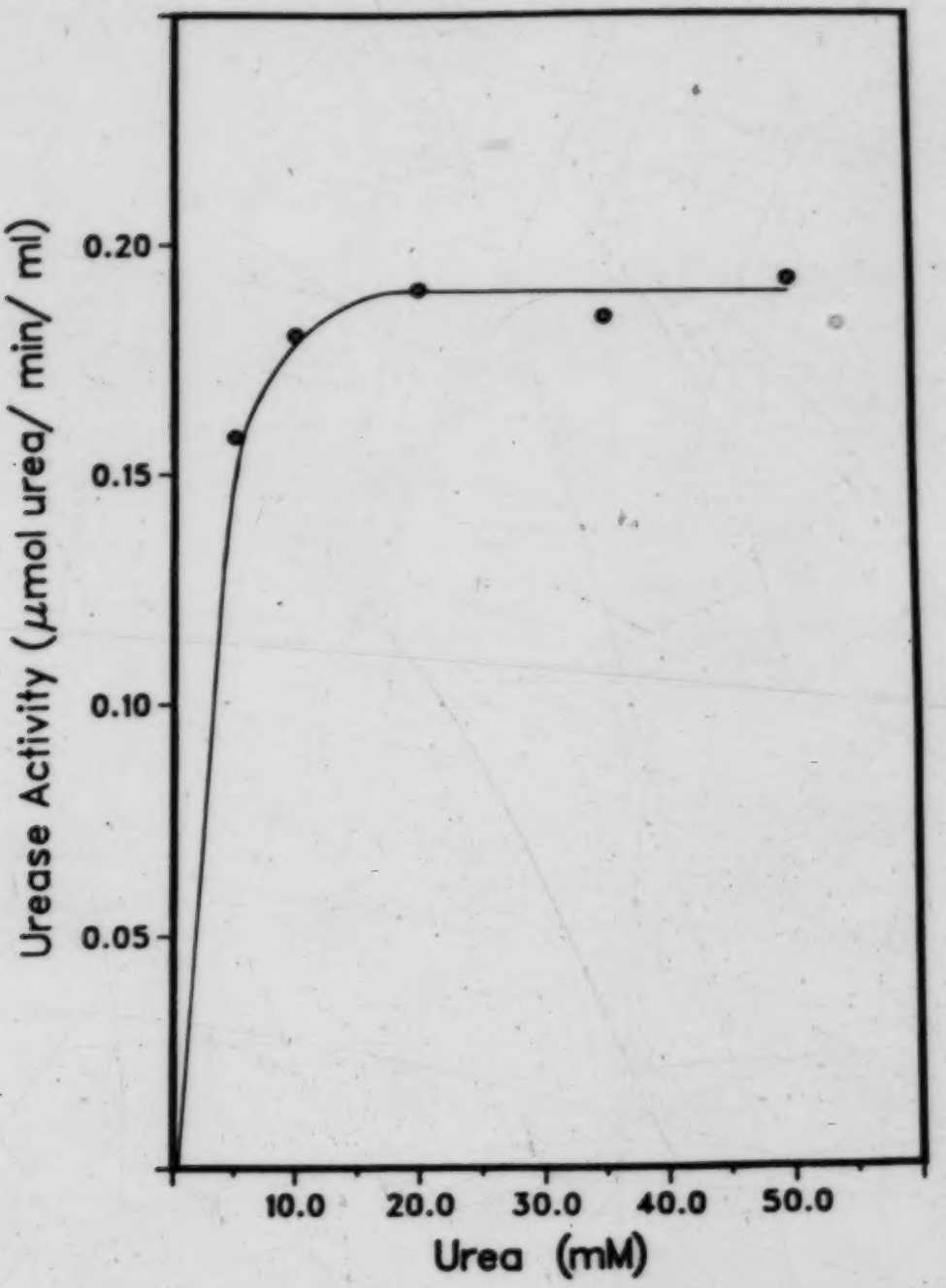
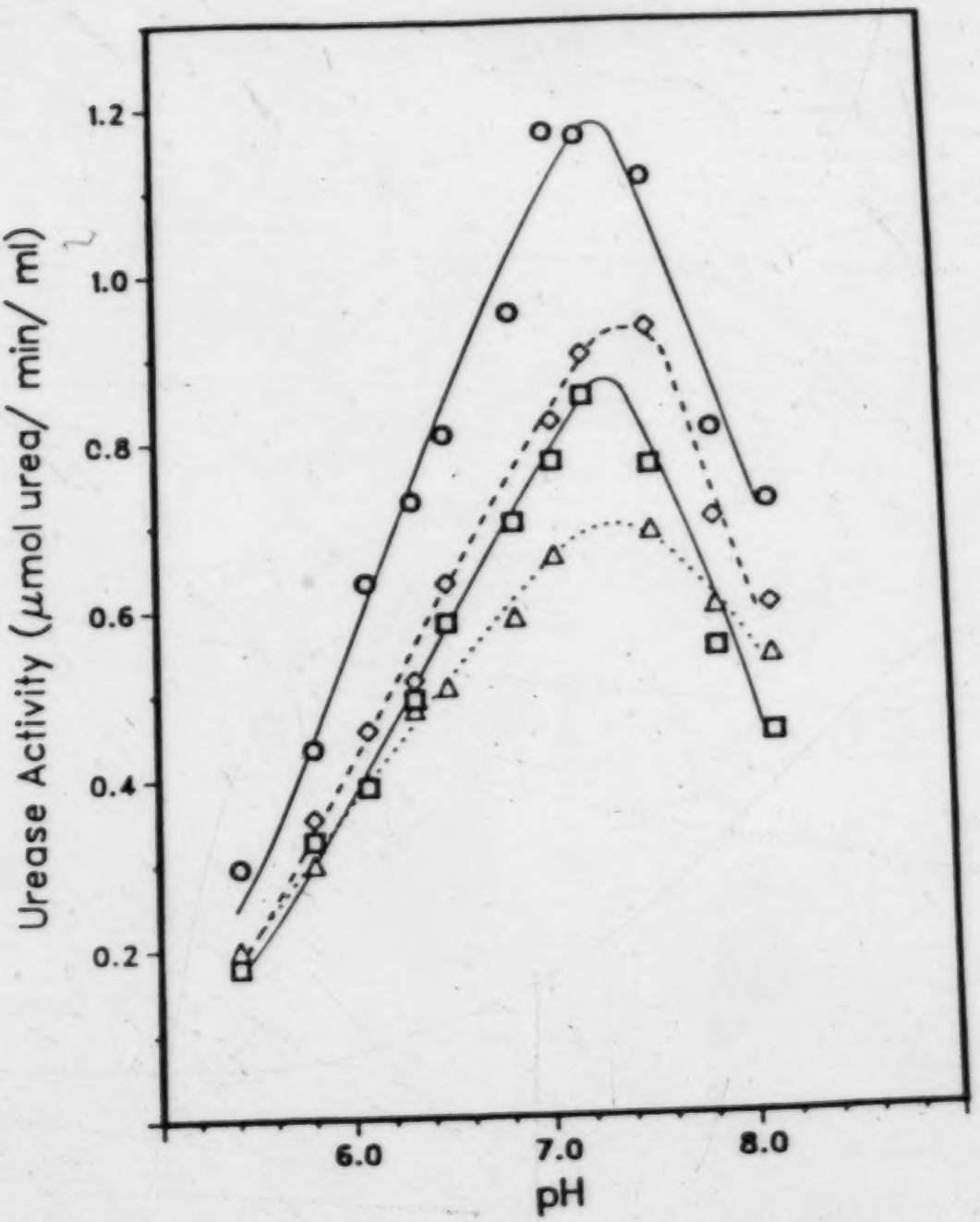

Urease Activity (µmol urea/ min/ ml)
0.20
0.15
0.10
0.05
10.0
20.0
30.0
40.0
50.0
Urea (mM)

Fig. 2. pH Optimum. Using the Berthelot assay, the enzymatic activity was followed as a function of pH from 5.5 to 8.0. The ureaplasma strains examined were: 1 (○), 3 (□), 8 (△), and 9 (◇).


Urease Activity (μmol urea/ min/ ml)
1.2
1.0
0.8
0.6
0.4
0.2
6.0
7.0
8.0
pH

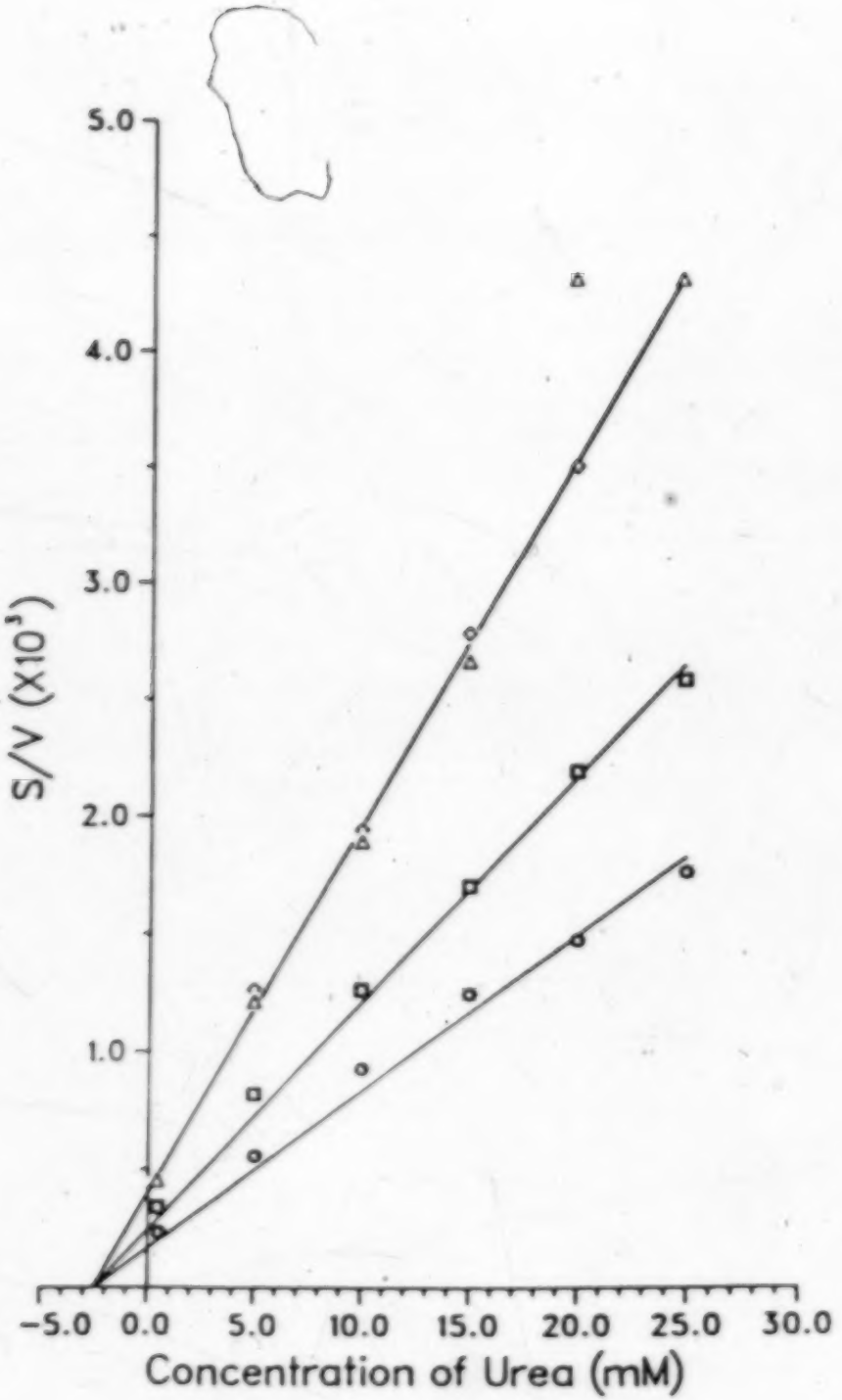
optimum was much greater than previously reported. The pH optimum for strain 8 was the same regardless if it was grown in Bromthymol Blue broth containing four times the concentration of urea (0.1% w/v), or whether the substrate solution contained 0.05 M HEPES buffer (as per Romano et al. (36)) instead of phosphate buffer (data not shown).

The specific activity of ureaplasma urease measured in µmol urea/ min/ mg total protein was determined as a function of urea concentration, and the data transformed to a plot of substrate/specific activity against substrate concentration. All four strains have a Km value of 2.5 mM urea, though the specific activity for each strain was different (fig. 3).

Immunoaffinity Column

When ureaplasma strain 8 cell lysate was passed through the immunoaffinity column, approximately 65% of the total recoverable urease activity bound (results not shown), with approximately 80% of the activity applied to the column being recovered. Urease that initially passed through the column failed to bind even after being repeatedly applied to the column. Bound urease could be eluted with 0.01 N NaOH. Elution with weak acid, e.g. 0.01 N HCl, rapidly destroyed enzymatic activity. The material that eluted from the column was judged as heterogeneous by SDS polyacrylamide gel electrophoresis. The specific activity of the material eluted from the immunoaffinity column was 0.8 times that of

Fig. 3. Km Determination. A plot of substrate/specific activity versus substrate concentration was used to determine the Km for ureaplasma urease. The x-intercept corresponds to -Km, and the slope is equal to the reciprocal of specific activity. The four strains examined were: 1 (○), 3 (□); 8 (△), and 9 (◇).


5.0
4.0
3.0
2.0
1.0
S/V (X10³)
-5.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
Concentration of Urea (mM)

the starting material.

Molecular Weight Determination

Urease activity was not observed with the Fishbein stain on either 0.75 or 1.5 mm thick SDS non-reducing polyacrylamide gels. Either the activity was below the sensitivity of the stain, or SDS inactivated the enzyme. Attempts to remove SDS and renature the enzyme by transferring to nitrocellulose also failed.

Although absolute electrophoretic mobility in native gel electrophoresis is determined by both surface charge and Stokes radius, a linear relationship between the relative mobility (Rf) of a protein and the polyacrylamide concentration can be obtained from the Ferguson plot (14) which has the relationship:

$$\log M = \log Mo - K T$$

where M is the mobility experimentally determined, Mo is the extrapolated mobility at zero acrylamide concentration, K is the slope constant, or retardation coefficient, and T is the polyacrylamide concentration. The slope value for a particular protein from the Ferguson plot is related to its molecular weight, and this relationship is used to establish a standard curve of retardation coefficients versus molecular weight.

The Ferguson plots (fig. 4 and 5) of ureaplasma urease and the molecular weight standards revealed a break from linearity at an approximate polyacrylamide concentration of

Fig. 4. Ferguson Plot of Ureaplasma Strains. Urease from ureaplasma strains 8 and 5 (O), 2(Δ), and 1, 3, and 6 (□) all exhibited a non-linear migration pattern at increasing concentrations of polyacrylamide. Hence, the region from 4 to 8% polyacrylamide concentration was chosen to determine to retardation coefficient.

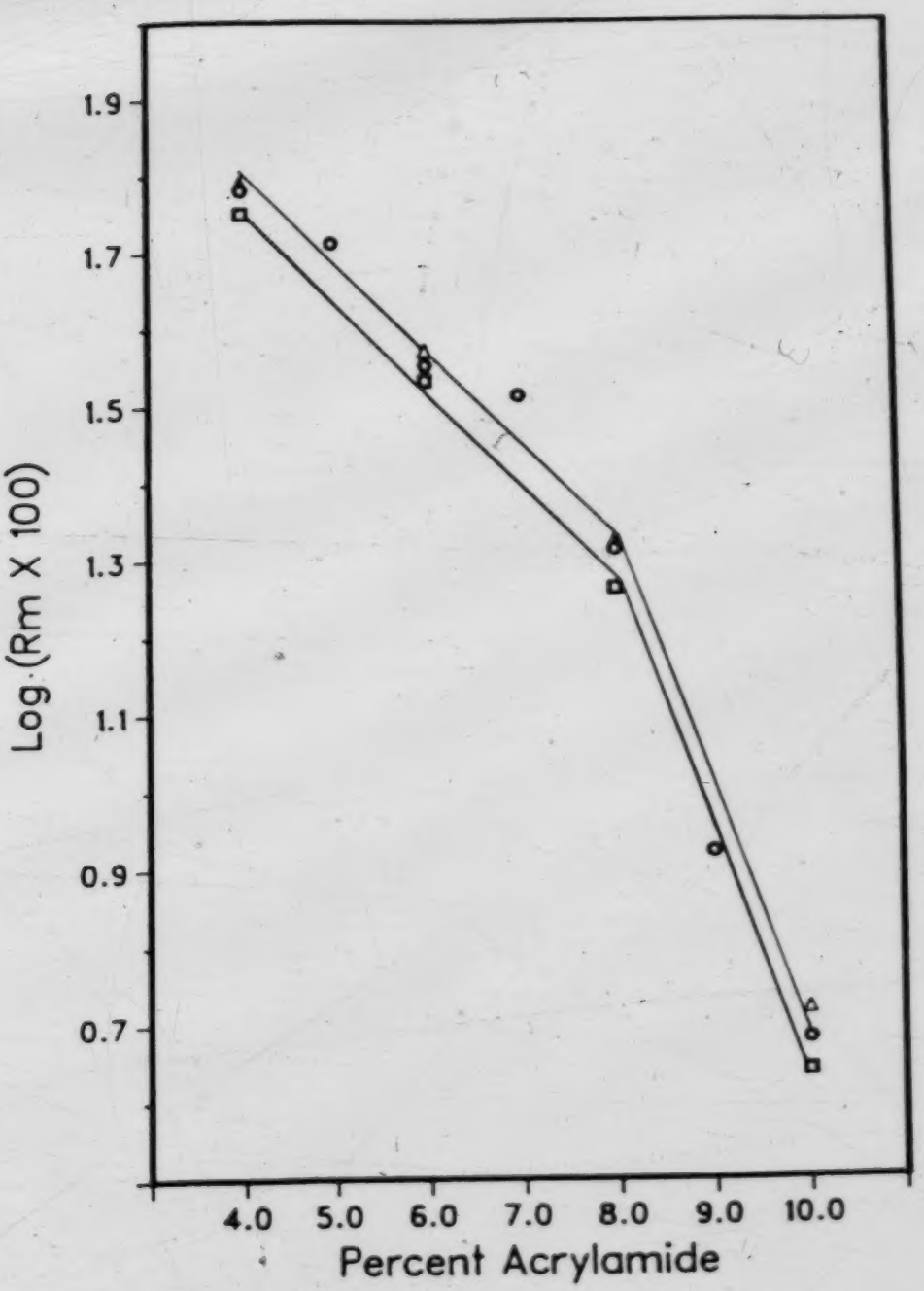
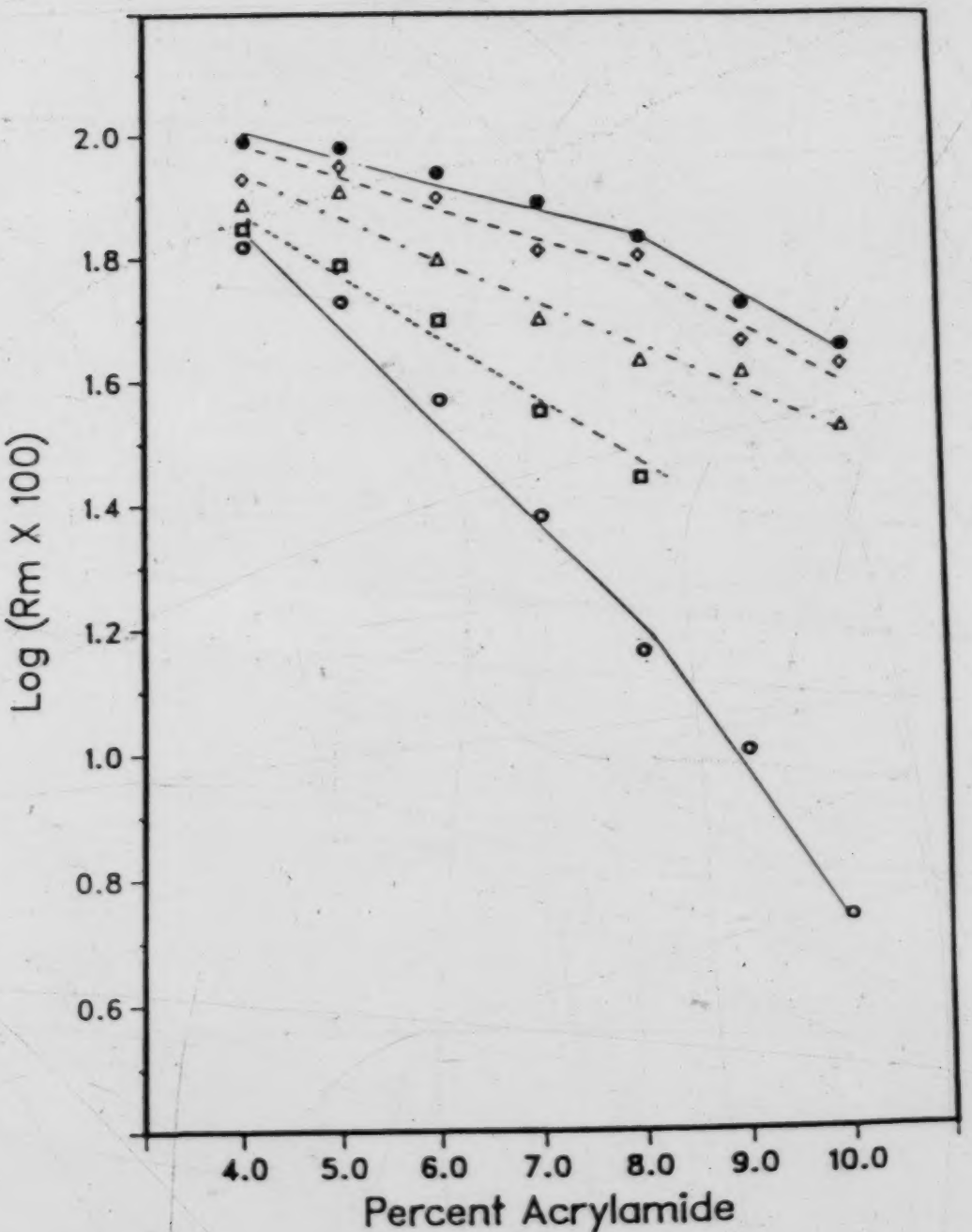

1.9
1.7
1.5
1.3
1.1
0.9
0.7
Log (Rm X 100)
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Percent Acrylamide

Fig. 5. Ferguson Plot of Molecular Weight Standards. A plot of the relative mobilities of standard molecular weight proteins as a function of acrylamide concentration. The molecular weight markers, and their corresponding molecular weights are as follows: jack bean urease dimer, 690,000 (O); jack bean urease monomer, 345,000 (□); myosin, 204,000 (△); phosphorylase, 116,000 (◇); and β-galactosidase, 97,000 (●).


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
Log (Rm X 100)
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Percent Acrylamide

8%, with the corresponding 9 and 10% ordinate values appearing low. Hence, only the region from 4 to 8% polyacrylamide concentration was used for regressional analysis to determine the retardation coefficients. Though strains 1, 3 and 6 migrated slower than strains 2, 5 and 8, the retardation coefficient of urease from all 6 ureaplasma strains was found to be -0.116, corresponding to an approximate molecular weight of 380,000 (fig. 6). By gel filtration on agarose beads, the molecular weight of strain 8 urease was found to be 330,000 (fig. 7).

Treatment with Sulfhydryl Reducing Agents

The effect of mercaptoethanol and dithiothreitol on ureaplasma urease was examined. Treatment of the cell lysate with sulfhydryl reducing agents for 2 hours at room temperature did not alter the relative electrophoretic mobility of enzymatically active urease, and there appeared to be no loss of enzyme due to dissociation, based on visual inspection. Alkylation by itself was found not to alter the relative mobility or to inhibit urease activity. Only after combined treatment with reducing agent and iodoacetamide was there a qualitative decrease of active urease compared to the untreated cell lysate.

Isoenzymes

Ureaplasma urease isoenzymes were not detected by the procedure of Delisle (8). After electrophoresis in either a

Fig. 6. Retardation Coefficients versus Molecular Weight. The relationship between the retardation coefficient and molecular weight for each molecular weight standard. The slope values were obtained by linear regression of the data from the Ferguson plot in the polyacrylamide concentration range of 4 to 8%. All ureaplasma strains examined had a slope of -0.116 (dashed lines).

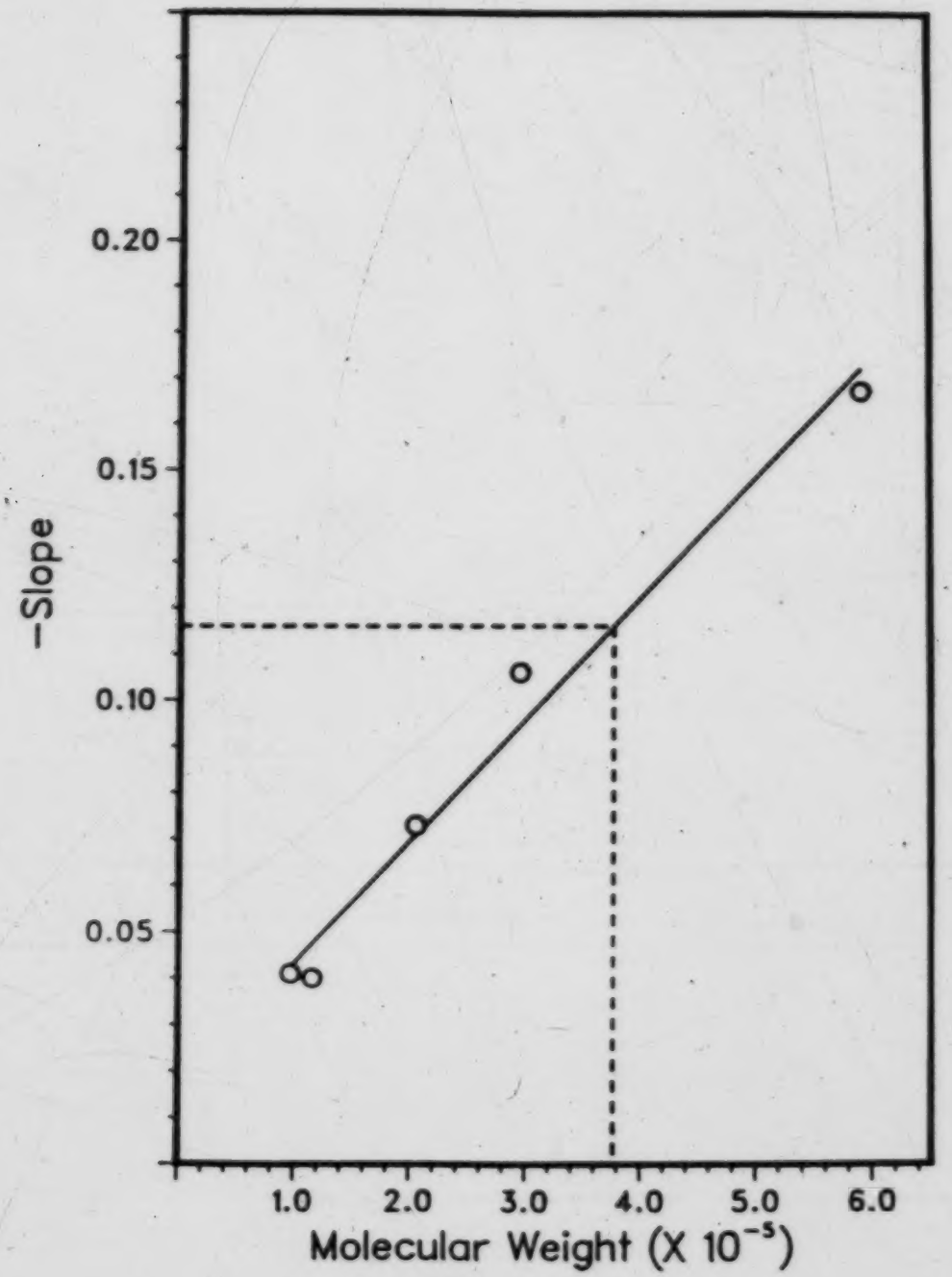
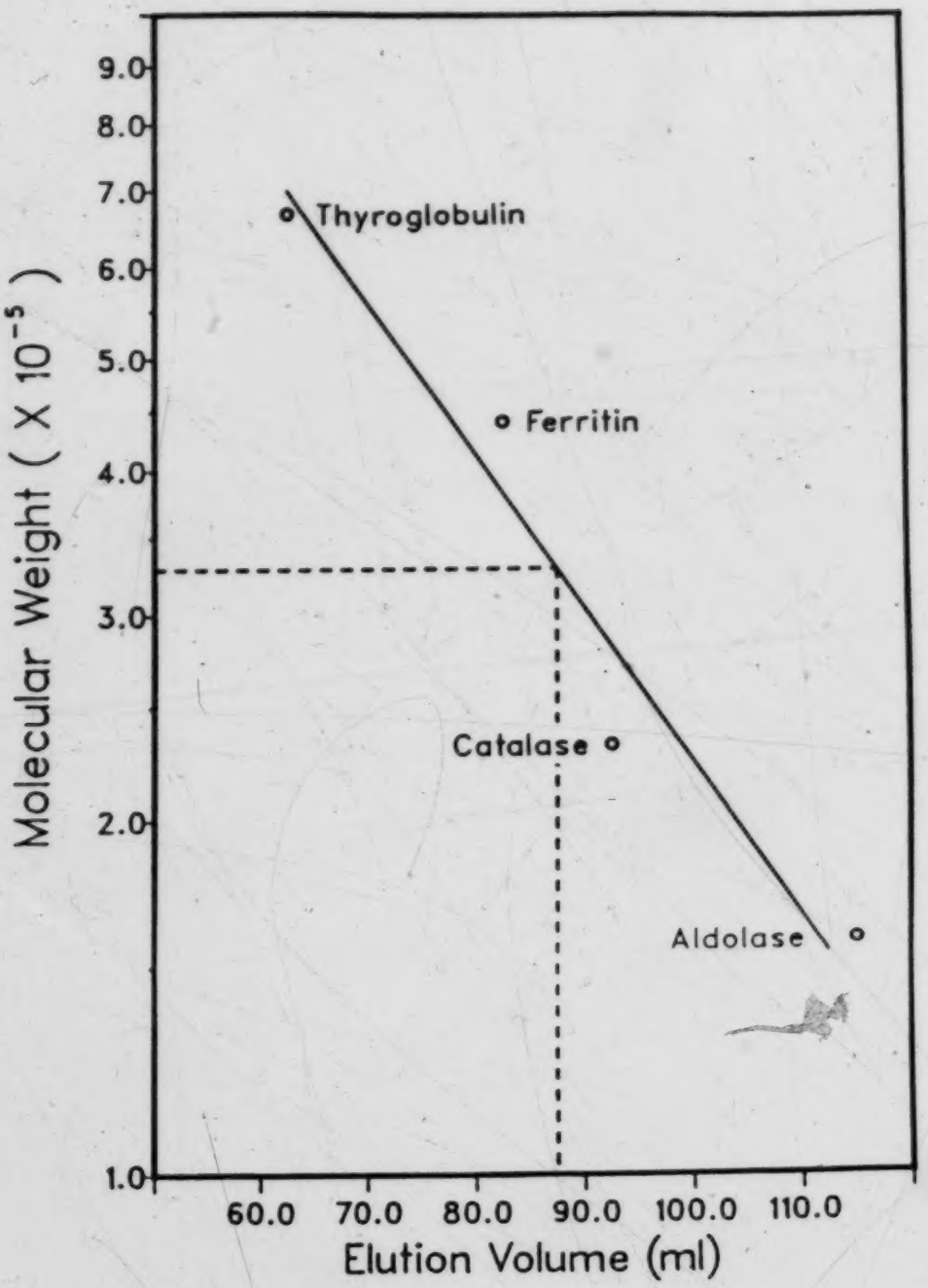

0.20
0.15
0.10
0.05
-Slope
1.0
2.0
3.0
4.0
5.0
6.0
Molecular Weight (X 10^{-5})

Fig. 7. Molecular Weight Determination by Gel Filtration. The molecular weight of ureaplasma urease was determined using a Sepharose 6B column as described in the text. Molecular weight markers included thyroglobulin (669,000), ferritin (440,000), catalase (232,000), and aldolase (158,000). Protein elution was detected at an absorbance of 280 nm, and urease activity was detected by the Berthelot assay. Ureaplasma urease was found to elute at approximately 86 ml, (dashed lines).


Molecular Weight (X 10^{-5})
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
Thyroglobulin
Ferritin
Catalase
Aldolase
60.0
70.0
80.0
90.0
100.0
110.0
Elution Volume (ml)

5% or 8% polyacrylamide gel, only a single band of active urease was observed. Delisle may have incorporated glycerol into the dilution buffer (a known dissociating agent for jack bean urease (2)) so the cell lysate was incubated in varying concentrations of glycerol (20, 50, and 90%) for 15 minutes prior to electrophoreses. Again, only a single band was observed with the same relative mobility as the untreated cell lysate. Urease isoenzymes were not observed after growth at a higher concentration of urea; urease from strain 8 cultured in Bromthymol Blue Broth containing either 0.1% or 0.025% urea yielded only one band with the same relative mobility.

Isoelectrofocusing

Multiple bands of urease activity were observed by isoelectrofocusing the cell lysate. A consistent distortion in the pH gradient ranging from 5.4 to 5.6 (fig. 8) had no effect on the results as the observed bands of urease activity were in a lower pH range. The number of bands observed for each strain, and its corresponding pH value, is given in Table 1. Six of the 7 ureaplasma strains examined had two visible bands of urease, while strain 8 had three. The majority of the bands were located near pH 5.2, with a range of 5.0 to 5.2.

Fig. 8. Isoelectrofocusing Gel pH Gradient. Shown is a representative pH gradient from a pH 4 to 6 isoelectrofocusing gel. There was a consistent disturbance in the gradient from pH 5.4 to 5.6. Active urease bands could be detected in the pH range of 5.0 to 5.2.

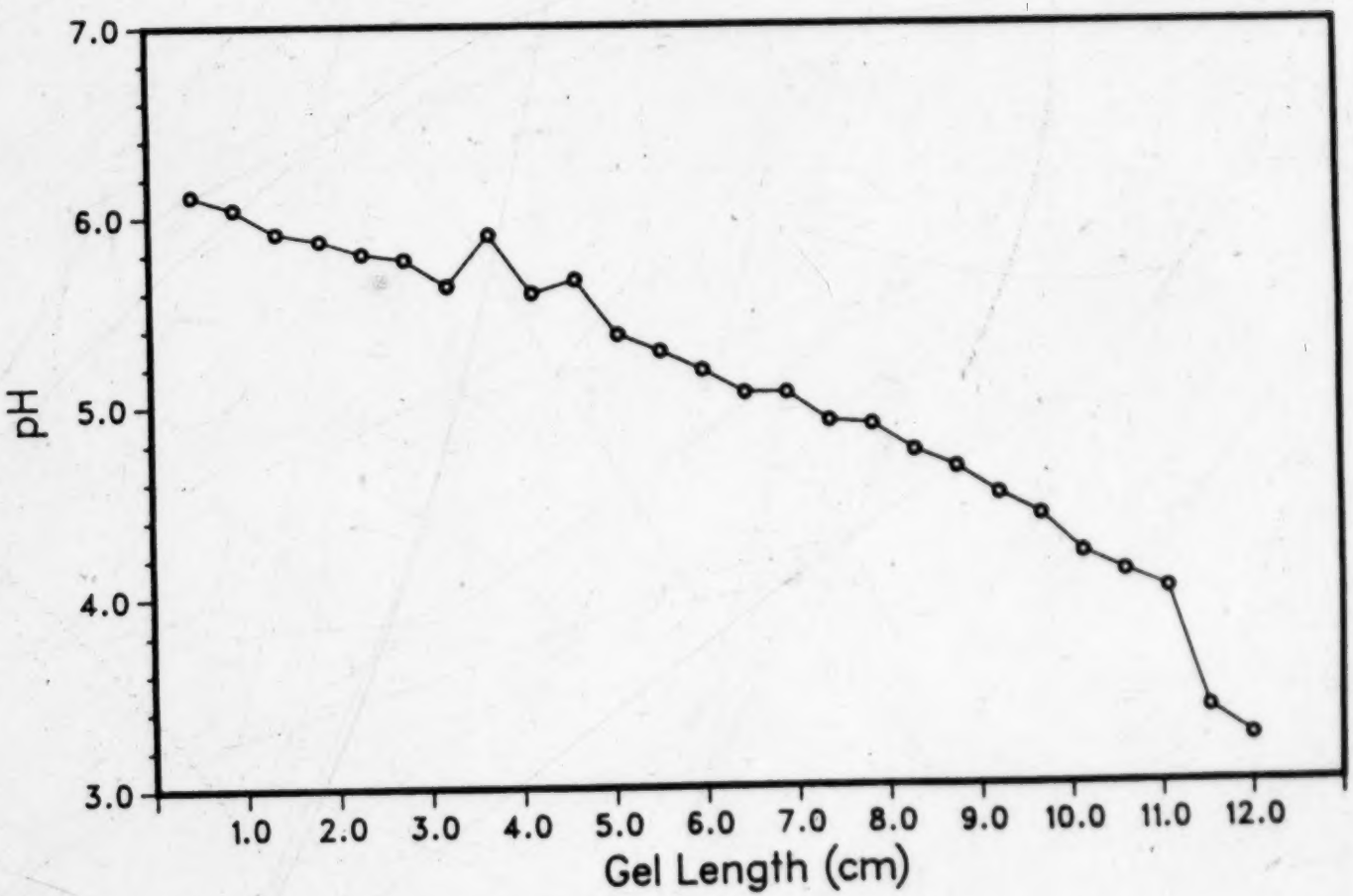

7.0
6.0
5.0
4.0
3.0
pH
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
Gel Length (cm)

Table 1. Summary of Isoelectrofocusing Results.

UREAPLASMA STRAIN	BAND[1]	Rf	pI[2]
1	1	0.49	5.2
	2	0.51	<5.2
2	1	0.48	5.2
	2	0.50	<5.2
3	1	0.50	5.2
	2	0.52	<5.2
4	1	0.48	5.2
	2	0.50	<5.2
5	1	0.48	5.2
	2	0.50	<5.2
6	1	0.46	5.0
	2	0.47	5.1
8	1	0,51	5.2
	2	0.53	<5.2
	3	0.54	5.1

[1]number of observable bands of urease activity detected by the Fishbein stain.

[2]pI of less than 5.2 indicates the pI of that band was below, but within experimental error of, 5.2.

D. Discussion

The pH optimum for ureaplasma urease has been reported (28, 38, 36) to range from 5 to 6, studying strains 6 and 8, while Swanberg et al. (40) reported pH optima at 5 and 7 with lower activity at pH 6 for strain 8. The pH optimum of the four ureaplasma strains examined here ranged from 7.2 to 7.5. Reasons for our values being considerably higher than previously reported values (28, 38) have not yet been determined. It has been suggested (personal communication to G. Stemke from N. Romano) that the optimum pH is a function of salt concentration. However, we found the pH optimum to be very similar whether urease was assayed in 0.1 M phosphate buffer or, as suggested by Romano et al. (36), in 0.05 M HEPES buffer. Although preferential induction of different sets of isoenzymes has been reported (19, 21), greater concentrations of urea during growth of strain 8 did not result in any detectable enzyme forms with a different pH optimum. All four strains examined had the same Km value of approximately 2.5 mM urea, agreeing with previously reported values (28, 36, 40). Different specific activity values for the individual strains may be due to variation in urease activity among strains, or in amount of urease or extraneous protein present in the cell lysate. The modified Berthelot assay was found to be extremely sensitive (to nmol quantities of ammonia) and linear through a wide range.

Unfortunately three attempts to purify ureaplasma urease failed and so the results were not presented, but

shall be discussed here. The first involved a one step purification process established for jack bean urease (7, 39, 43). The purification utilized an affinity column consisting of hydroxyurea (a substrate analog for jack bean urease), coupled to Sepharose 4B (Appendix A). Synthesis of the affinity column was successful as jack bean urease bound to the column. However, in my hands, ureaplasma urease failed to bind, possibly because coupled hydroxyurea was no longer a suitable substrate analog. The second attempt, an immunoaffinity column using rabbit anti-strain 8 urease antiserum, bound approximately 65% of the total recoverable activity. It is possible that modifications such as proteolytic digestion, may have destroyed the epitope(s) in 35% of the urease population. To explain the drop in specific activity of the immunoaffinity purified material, it is possible that the alkaline conditions used for elution may have slowly denatured the enzyme. Equally possible is that the immunoaffinity column either bound cross reactive cellular material, or non-specifically bound cellular material. The drop in specific activity may also indicate that the antiserum is not specific for ureaplasma urease. An attempt at partial purification by separation on the basis of Stokes radius (Sepharose 4B), and then by charge (DEAE) was not successful; the specific activity of the semi-purified material was only 1.5 times greater than that of the starting material, with recovery being only 20%. From isoelectric focusing results the pI of ureaplasma

urease was approximately 5.2, hence urease should have bound to the DEAE column at pH 7.2. It is possible, though, that the concentration of the counterion (0.1 M phosphate) was too high to permit the binding of urease.

An affinity column using a different substrate analog, such as fluorofamide, may prove to be an effective one step purification procedure as one may be able to couple fluorofamide without significantly altering the binding site. Generation of monoclonal antibodies against a ureaplasma urease epitope that was on the surface of the antigen, and synthesis of a specific immunoaffinity column would also permit rapid and convenient isolation. As one can select the clones that are synthesizing antibodies to urease, purified urease is not required. Alternatively, cloning the gene for urease into a bacterium to produce pure urease in large quantities can also be attempted. Purification using classical methods might be possible if enough cell material could be obtained, perhaps by using continuous culture methods (3, 26).

Once the enzyme has been purified, a multitude of experiments may be contemplated. Of particular interest would be to determine if ureaplasma urease is a metalloenzyme using spectrophotometric analysis (9, 10). As well, examination of the specific activity values from the different strains, to determine if they represent catalytically variant forms of ureaplasma urease, could be done.

. The Ferguson plots for ureaplasma urease (fig. 4), and to a lesser extent for the molecular weight standards (fig. 5), reveal a biphasic relationship. This suggests retardation of migration probably resulting from a protein with a large Stokes radius trying to penetrate a gel of relatively small pore size. Such retardation of migration has been observed by Raymond and Nikamich (32), and by Zwann (44) with proteins of lower molecular weight than that used in this study, but at polyacrylamide concentrations greater than 10%.

The molecular weights of urease from six ureaplasma strains were found to be approximately 380,000 by native gel electrophoresis. For strain 8 a molecular weight of 330,000 was determined by molecular sieving. These values are much larger than those reported by Kenny (22) though his data was not presented. Although urease from all 6 strains exhibited the same slope from the Ferguson plot, and hence the same molecular weight, strains 1, 3 and 6 had a different relative mobility than strains 2, 5 and 8. Based on the work of Hedrick and Smith (18), such parallel slopes suggests urease from strains 1, 3 and 6 are charge isomers of 2, 5 and 8. Hence the two forms of urease have a similar molecular weight, but a difference in net charge results in the difference in mobility. This lends further support to taxonomic studies based on hybridization experiments with DNA (5), and polyacrylamide gel elecrophoretic comparison of polypeptides (20) which have shown that the first 8 serovars

may be divided into two groups composed of stains 1, 3, and 6, and of strains 2, 4, 5, 7, and 8.

Given the large molecular weight of ureaplasma urease, the presence of subunits is expected. Neither the migration rate, nor the qualitative amount of ureaplasma urease present, appeared to change in the presence or absence of either mercaptoethanol or dithiothreitol. Disulfide bonds may have been reduced, but strong ionic or other non-covalent interactions may have maintained the integrity of the enzyme. Such molecular stability due to non-covalent interactions is also found with immunoglobulins, for example, where reduction without denaturation results in intact immunoglobulins (12). Only upon treatment of the cell lysate with reducing agent and iodoacetamide (in the absence of denaturing agents) was there loss of active urease, though without any concomitant increase in the number of visible active urease bands. Loss of enzymatic activity after reduction and alkylation could have resulted from either: 1) an inactive holoenzyme, where a change in conformation either destroyed, or buried the catalytic site within the enzyme, 2) the enzyme dissociated into several active subunits that were not detected due to the insensitivity of the stain, or 3) the enzyme dissociated into subunits with subsequent destruction of the catalytic site. Which of these possibilities is correct can not be determined from this analysis, though results presented later may offer some insight into this question.

Overestimation of molecular weight occurs with glycoproteins in SDS polyacrylamide gel electrophoresis, as the migration rate of glycoproteins is not proportional to molecular mass (37, 4). As well, the presence of carbohydrate side chains may affect the conformational integrity of the protein as has been shown for porcine pancreatic RNase (42) and rabbit IgG (23). Hence it is important to determine if ureaplasma urease possesses carbohydrate side chains. Such a determination may be accomplished by staining the gels with carbohydrate specific stains.

Delisle (8) and Romano et al. (36) have reported the presence of urease isoenzymes. There are numerous definitions for an isoenzyme, but the following has been adopted: isoenzymes are enzymes with the same catalytic ability, but which have arisen from distinct structural genes, and are distinguishable by their physio-chemical properties (30). Using this definition, isoenzymes are distinct from multiple enzyme forms that resulted from post-translational or other modifications (such as proteolytic digestion).

Only a single band of active urease was detected using the procedure of Delisle (8), regardless of the urea concentration during growth, which might induce formation of different forms of urease, or the presence of glycerol during electrophoresis. This result agrees with work done on molecular weight determination using native gel electrophoresis, where again only a single band was observed. Since Delisle examined ureaplasmas from clinical isolates, the

multiple bands may have been a phenomenon of the isolates, e.g. perhaps multiple strains were present, and not reproducible with stock strains. The isoenzymes reported by Romano et al. (36) using a similar electrophoretic procedure may have been due to the acetone treatment. It remains possible that multiple enzyme forms exist, but were not resolved by the procedure used.

Multiple bands of active urease were observed upon isoelectrofocusing the cell lysate. The pI of the multiple bands ranged from 5.0 to 5.2, with the separation between bands usually being less than 0.1 pH units. As the pattern of active urease bands for a given strain was reproducible over time, it is unlikely that they are due to slow proteolysis or other *in vitro* modifications (e.g. slow de-amidation). The small charge differences between the multiple forms of urease are likely beyond the resolving power of native gel electrophoresis, hence only a single band of urease activity was observed.

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III. Immunological Characterization

A. Introduction

Antibody-enzyme interactions may result in changes in enzymatic activity. The outcome of antibody-enzyme interactions on enzyme activity varies from neutralization (or reduction (1)), to enhancement (22, 36). The extent of neutralization is often related to substrate size (35, 4, 7, 12), indicating that the antibody sterically hinders entrance of the substrate to the catalytic site. Hence, for low molecular weight substrates, the extent of neutralization is small, while for macromolecular substrates the degree of neutralization is large. However, antibodies may also neutralize enzymatic activity by combining with the catalytic site (11), or by inducing conformational changes (33). Immunological studies on jack bean urease (21, 25) have shown 66 to 78.5% antibody neutralization levels. Such a high level of neutralization for a low molecular weight substrate suggests antibodies are either inducing conformational changes, or reacting with the catalytic site itself.

Specific antisera can be used to detect similarities between proteins by the degree of cross reactivity. A correlation between variation in primary amino acid sequence and immunological cross reactivity has been shown for cytochrome c (24). Cross reactivity of particular proteins from different species has also been related to the

phylogenetic distance between the species compared (1, 34, 38). Principles of cross reactivity can be extended to antibody neutralization of isofunctional enzymes, where the extent of neutralization is related to phylogenetic distance, and homology in amino acid sequence (3). Using antiserum generated against *Ureaplasma urealyticum* strain 8 urease, the cross neutralization between other strains of ureaplasmas, as well as other bacterial ureases, was evaluated. It has been suggested (27, 23, 42) that a number of mycoplasmas have undergone degenerative evolution from true bacteria. Cross neutralization studies comparing ureaplasma urease and other bacterial urease, were undertaken in the hope of gaining insight into the evolutionary background of *Ureaplasma urealyticum*.

After electrophoretic transfer of protein to nitrocellulose, the immunoblot procedure utilizes specific antiserum to probe for an enzyme, thereby obviating the need for a specific enzymatic stain. As many biochemical procedures inactivate enzymes but do not destroy antigenic reactivity, one way to detect the enzyme would be to use specific antiserum. This technique was utilized to further characterize ureaplasma urease.

B. Materials and Methods

Materials

All bacterial culture media, gelatin, and Freund's adjuvant were purchased from Difco. Jack bean urease and hydrogen peroxide (30%) were obtained from Sigma. Ethyl-chloroformate was from Eastman Kodak, horse serum from Flow Lab., and immunodiffusion cells were from Cordis Lab..

Sources of Urease

Ureaplasma urealyticum strains 1 through 5 and 8 were harvested and lysed as described in Chapter II, and stored at -70°C. Three animal ureaplasmas - avian, bovine, and simian (supplied by Dr. Robertson, Med. Micro., Univ. of Alberta, Canada) - were also examined.

Other sources of urease included jack bean urease and several bacteria (Table 2). All bacterial species except *Clostridium histolyticum* were grown in tryptic soy broth with vigorous aeration at 37°C overnight. When the optical density of the culture at 600 nm was 1.0, cells were collected by centrifugation in the Sorvall GSA head at 16,000 g for 10 minutes. The pellet was resuspended in a minimum volume of saline and lysed in a French press (American Instrument Co.) at 20,000 psi. *C. histolyticum* was cultured in fluid thioglycolate medium under anaerobic conditions at 37°C for 4 days before collecting and lysing the cells as above.

Table 2. Urease-Producing Bacterial Species Examined.

GRAM STAIN	BACTERIAL STRAIN
Positive	*Clostridium histolyticum*
	Staphylococcus aureus
Negative	*Proteus vulgaris*
	Pseudomonas aeruginosa
	Providentia rettgeri
	Klebsiella aerogenes

Antisera Production

Rabbit antiserum against whole *Ureaplasma urealyticum* strain 8 cell lysate (supplied by Dr. Stemke, Dept. Micro., Univ. of Alberta, Canada, and prepared as described in (31)) was used in two-dimensional immunoelectrophoresis (41). The immunoprecipitate band corresponding to ureaplasma urease was identified by the Fishbein stain (15). After extensively washing in saline, the immunoprecipitate band was cut out and a complete Freund's adjuvant of the ureaplasma urease/anti-urease precipitates was prepared (8). Rabbits were immunized with 1.5 ml of adjuvant subscapularly and a total of 0.25 ml in the toe pads of each hind leg (estimated amount of urease per immunization is approximately 24 µg). This was repeated twice at six week intervals followed by exsanguination four weeks after the last injection.

Specificity of the antiserum was examined using an immunodiffusion cell and two-dimensional immunoelectrophoresis against ureaplasma strain 8 whole cell lysate. The antiserum reacted with one component from ureaplasma cell lysate, and another from horse serum (probably a contaminant from the growth medium). Removal of rabbit anti-horse serum antibodies was accomplished by passing the antiserum through a column of ethylchloroformate crosslinked horse serum (6). Preparation of the column involved the dropwise addition of 0.06 ml ethylchloroformate per ml horse serum that had been dialyzed overnight against 0.2 N sodium acetate buffer pH 4.5. The pH of the reaction mixture was maintained

between 4.5 and 5.0 by the addition of 1 M NaOH. One hour after coagulation, the solidified protein was homogenized to a fine suspension and sequentially washed with 3 litres of PBS, 200 ml of 0.1% sodium carbonate, 300 ml of PBS, and 0.2 M glycine/HCl pH 2.2. The crosslinked protein was then equilibrated with PBS and placed into a 10 ml column. When the absorbed rabbit antiserum was re-examined by two-dimensional immunoelectrophoresis against strain 8 lysate, only the immunoprecipitate band corresponding to ureaplasma urease was detected. For future reference, the rabbit anti-ureaplasma strain 8 antiserum thus obtained shall be referred to as "anti-urease antiserum".

Antibody Neutralization Assay

Anti-urease antiserum was diluted 10 fold from 1:100 to 1:100,000, and from 1:500 to 1:500,000 with a solution of bovine serum albumin (5 mg/ml in 10 mM EDTA and 0.2 M phosphate buffer pH 7.2). The control consisted of normal serum at 1:100 in the same diluent. Equal volumes of appropriately diluted ureaplasma or bacterial cell lysate and each of the antiserum dilutions were combined, and incubated overnight at 4°C in a capped microfuge tube. Residual urease activity in 100 µl of the cell lysate - antiserum mixture was determined by the modified Berthelot assay as described in Chapter II.

Immunoblot

All steps in the immunoblot procedure were performed at room temperature. After the proteins were electroblotted, the nitrocellulose was immersed first in 0.05 M citrate buffer (pH 6,0) for 15 minutes, then in a solution of 20 mM Tris-HCl buffered saline pH 7,5 (TBS) containing 3% gelatin for 30 minutes. The gelatin solution was replaced by anti-urease antiserum, diluted 1:100 in TBS, and incubated overnight. Next morning the nitrocellulose was washed twice for 20 minutes each with TBS containing 0.05% Tween 20 (TTBS). Goat anti-rabbit IgG antiserum conjugated to horse-radish peroxidase (HRP, Cappel) diluted 1:2000 in TBS was allowed to react for 5 hours. This was followed by two washes in TTBS as above. The color development solution was prepared fresh and consisted of 1) 60 µg BioRad HRP color development reagent (4-chloro-1-napthol) in 10 ml ice cold methanol, and 2) 100 µl of 30% hydrogen peroxide in 100 ml TBS at room temperature. The dissolved color development reagent was combined with the peroxide solution and added to the nitrocellulose. Color development required 15 to 45 minutes, and was terminated by several washes with water.

C. Results

Antibody Neutralization of Urease Activity

The Berthelot assay was used to detect residual urease activity, and the extent of neutralization was determined by

the following formula:

$$\frac{\text{Maximum Enzyme Activity} - \text{Minimum Enzyme Activity}}{\text{Maximum Enzyme Activity}} \times 100$$

The greatest concentration of antiserum which could be examined was a 1:100 dilution as the color of the serum interfered with the color of the Berthelot assay. Neutralization data for all sources of urease examined are shown in figures 9 to 13, and summarized in Table 3. Inhibition of urease from human ureaplasma strains ranged from 17% (for the homologous strain 8 urease) to 57%, while neutralization of animal strains ranged from 44 to 73%. Even at high concentrations of anti-urease antiserum, the bacterial and jack bean ureases examined were not neutralized.

Immunoblot of Non-Denaturing, Non-Reducing Gels

Specificity of the anti-urease antiserum was re-examined by polyacrylamide gel electrophoresis. After electrophoresis of strain 8 cell lysate, horse serum, and Bromthymol Blue broth in a native polyacrylamide gel, the protein was electroblotted to nitrocellulose, and detected by the immunoblot procedure. Antibodies bound to two components in the strain 8 cell lysate, but not to horse serum or medium components (fig. 14). Of the two bands detected in strain 8 lysate, only the faster migrating band corresponded to the band of urease detected by the Fishbein stain (fig. 15). The identity of the slow moving band is uncertain.

Fig. 9. Neutralization of Ureaplasma Urease by Anti-Urease Serum: Less than 40% neutralization. Ureaplasma strains 1 (□) and 8 (○).

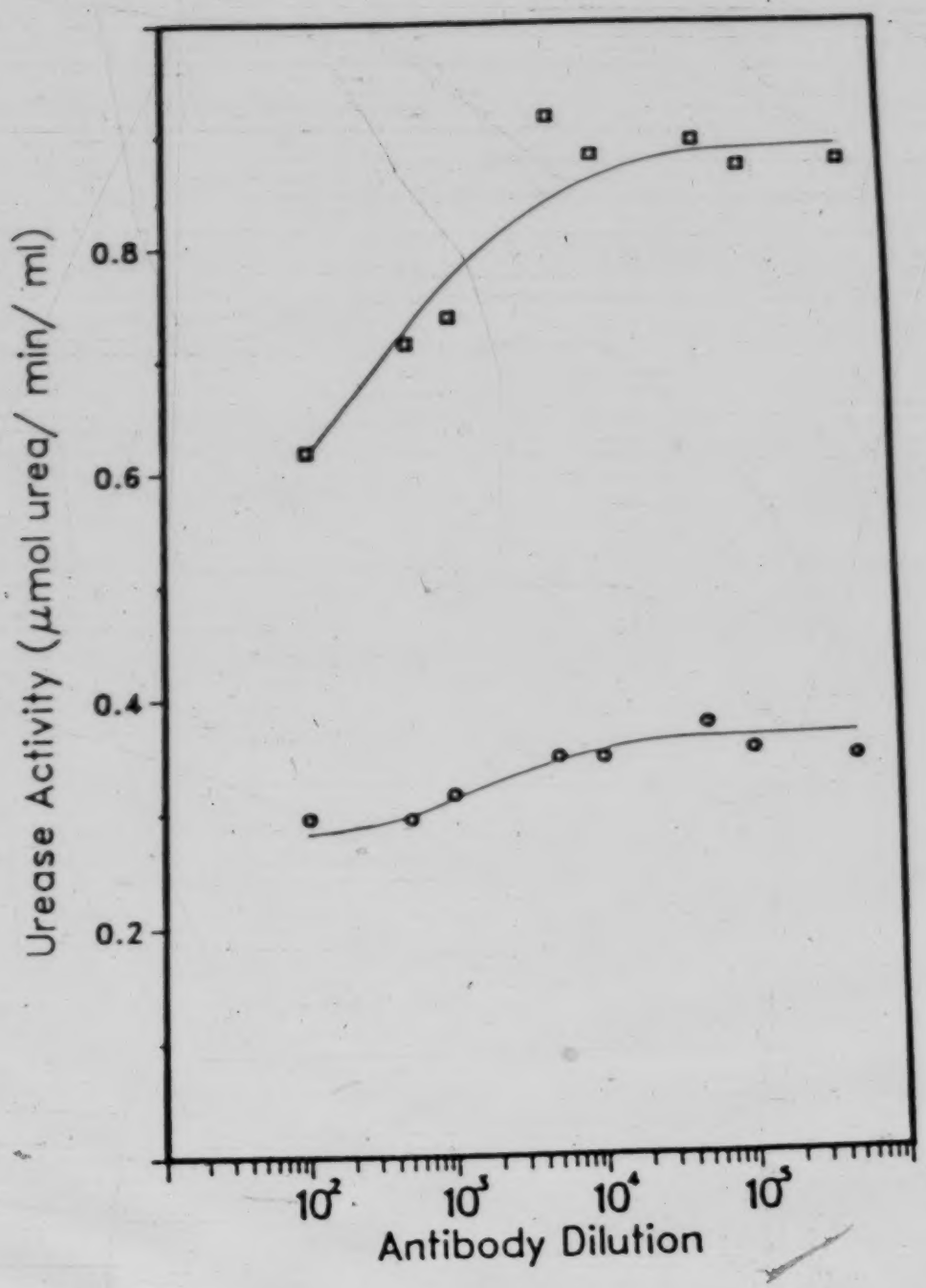

Urease Activity (μmol urea/ min/ ml)
0.8
0.6
0.4
0.2
10²
10³
10⁴
10⁵
Antibody Dilution

Fig. 10. Neutralization of Ureaplasma Urease by Anti-Urease Serum: Between 40 and 60% neutralization. Ureaplasma strains examined included 2 (O), 3 (Δ) 4 (●), and 5 (□).

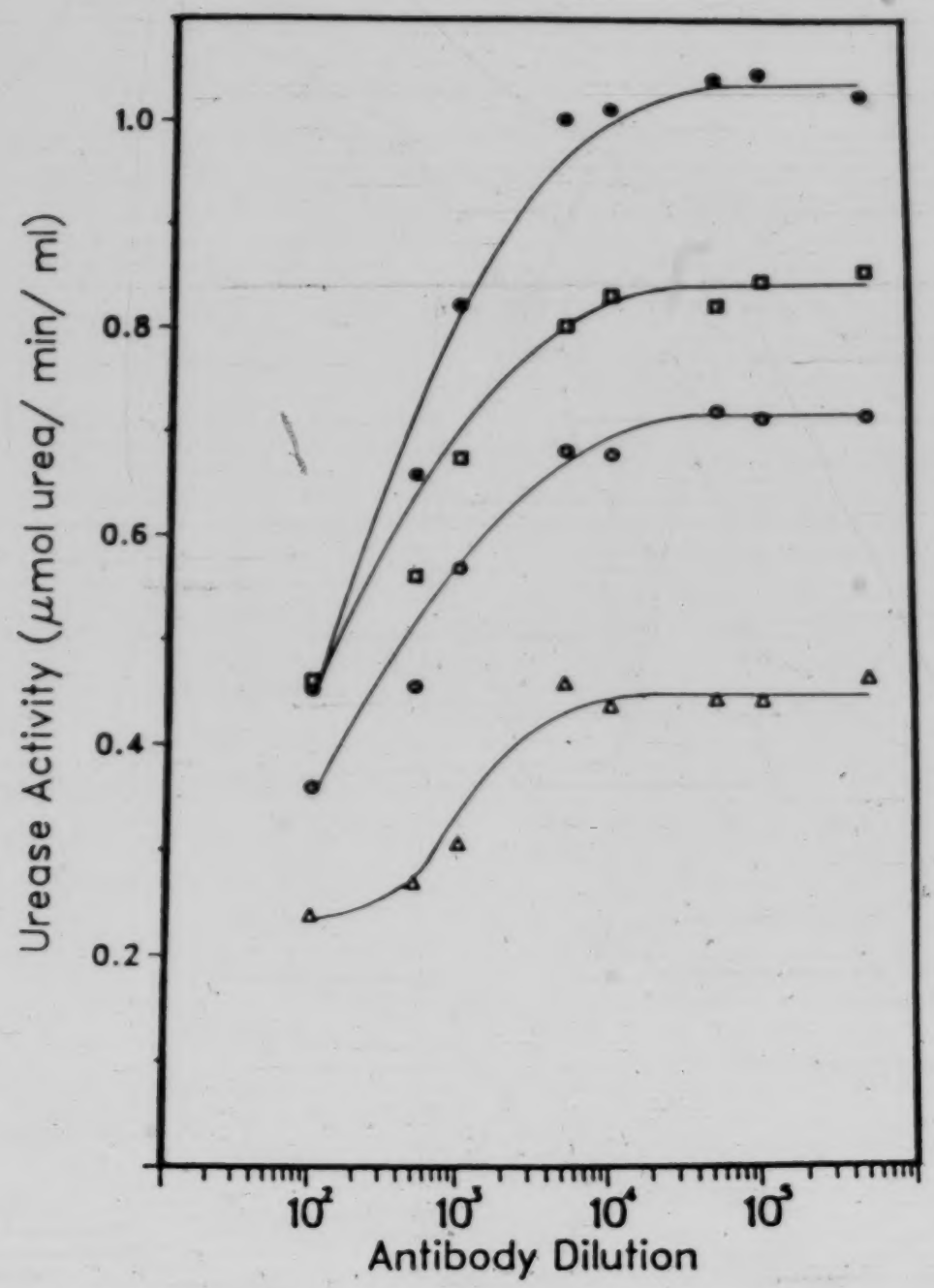

Urease Activity (μmol urea/ min/ ml)
1.0
0.8
0.6
0.4
0.2
10²
10³
10⁴
10⁵
Antibody Dilution

Fig. 11. Neutralization of Ureaplasma Urease by Anti-Urease Serum: Greater than 60% neutralization. Urease of the avian (O), simian (Δ) and bovine (□) strains.

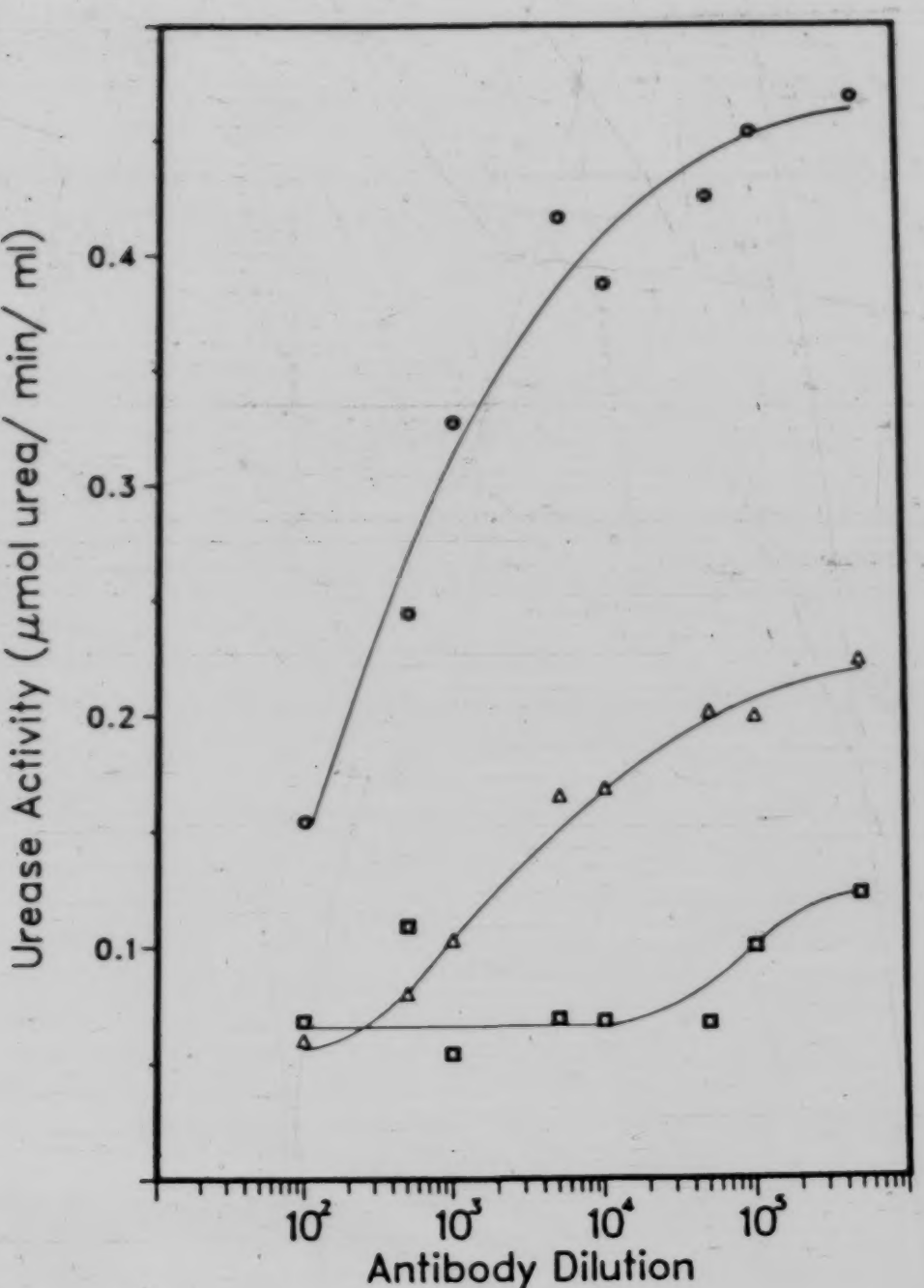

Urease Activity (μmol urea/ min/ ml)
0.4
0.3
0.2
0.1
10²
10³
10⁴
10⁵
Antibody Dilution

Fig. 12. Neutralization of Urease from Gram Positive Bacteria and Jack Bean. Affects of anti-strain 8 urease antiserum on *S. aureus*(□), *C. histolyticum* (○), and jack bean urease (△).

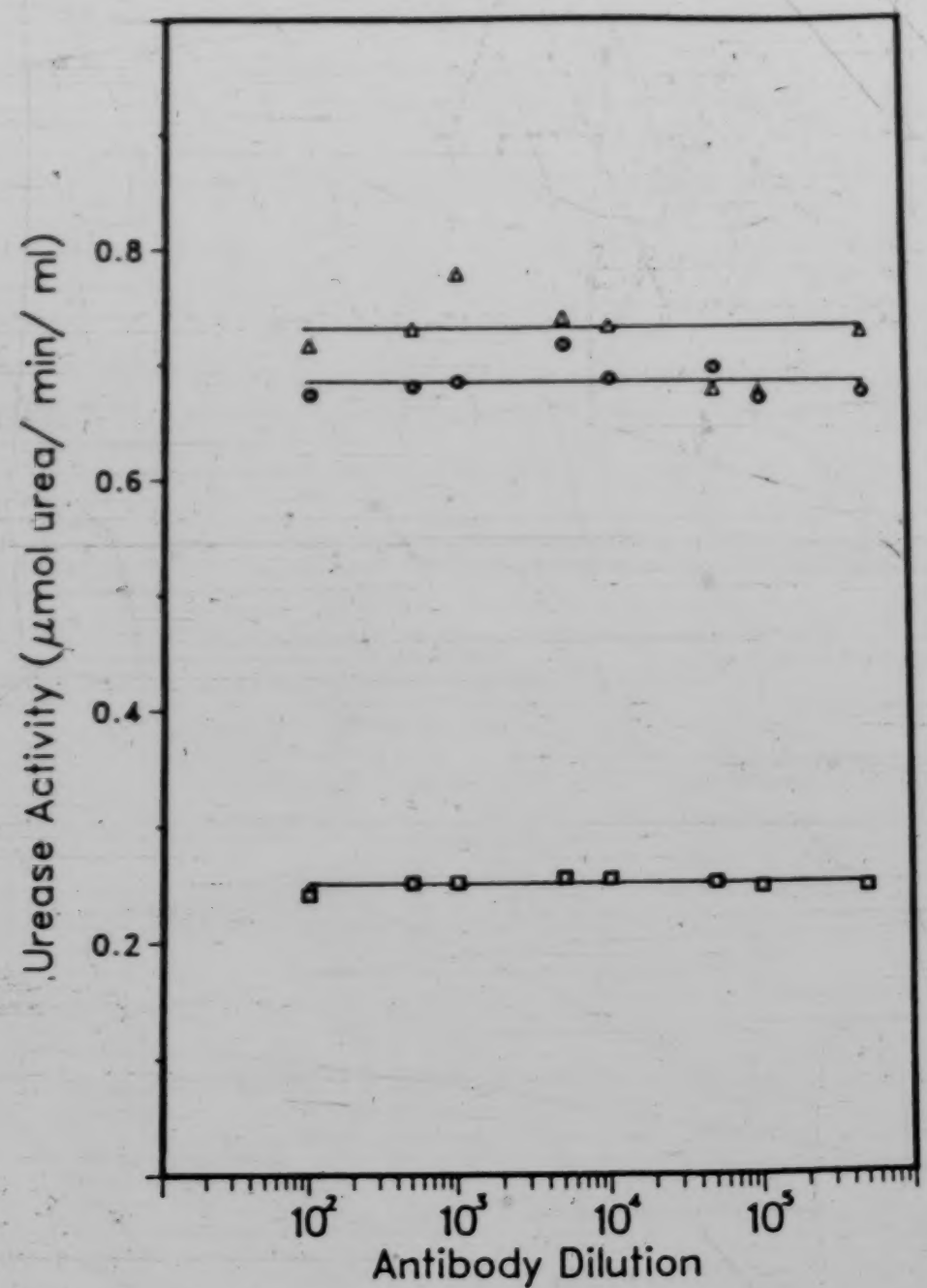

Urease Activity (μmol urea/ min/ ml)
0.8
0.6
0.4
0.2
10²
10³
10⁴
10⁵
Antibody Dilution

Fig. 13. Neutralization of Urease from Gram Negative Bacteria. Affects of anti-strain 8 urease antiserum on the following Gram negative bacteria: *P. rettgeri* (O), *P. vulgaris* (□), *P. aeruginosa* (Δ), and *K. aerogenes* (●).

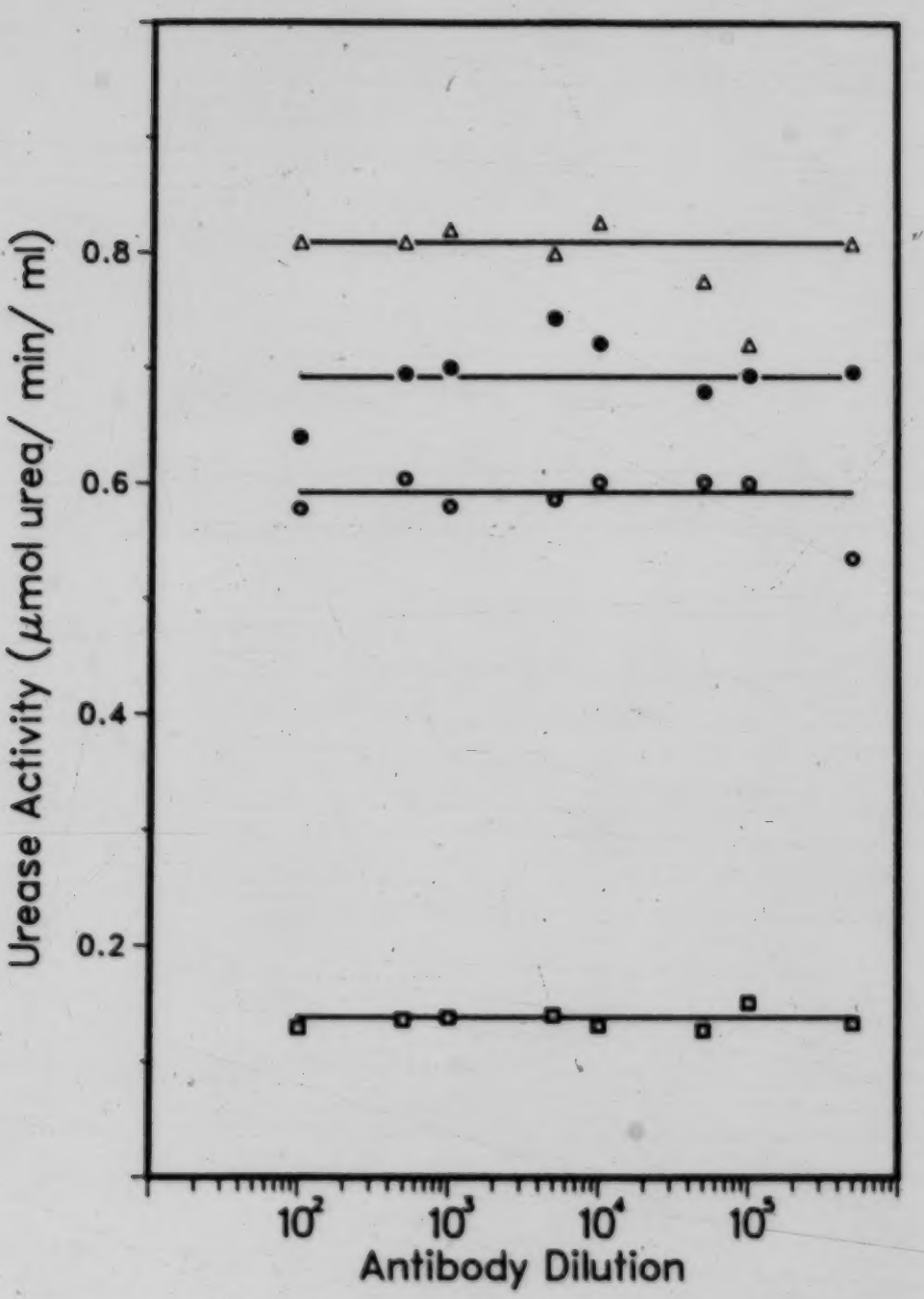

Urease Activity (μmol urea/ min/ ml)
0.8
0.6
0.4
0.2
10²
10³
10⁴
10⁵
Antibody Dilution

Table 3. Neutralization by anti-strain 8 urease antiserum.

ORGANISM		% INHIBITION[1]
Ureaplasma strain	1	31
	2	49
	3	56
	4	57
	5	47
	8	17
	Avian	67
	Bovine	44
	Simian	73
Gram positive bacteria	*C. histolyticum*	<10
	S. aureus	<10
Gram negative bacteria	*P. vulgaris*	<10
	P. aeruginosa	<10
	P. rettgeri	<10
	K. aerogenes	<10

[1]neutralization values of less than 10% indicate that neutralization was negligible, but that the scatter was less than 10%.

Fig 14. Specificity of the anti-Ureaplasma Urease Antiserum. Shown is nitrocellulose from a blot of strain 8 cell lysate (lane 1), and horse serum (lane 2) subjected to electrophoresis in an 8% non-reducing, non-denaturing polyacrylamide gel. Approximately 15 µg of strain 8 cell lysate and 40 µg of horse serum were applied to the column.

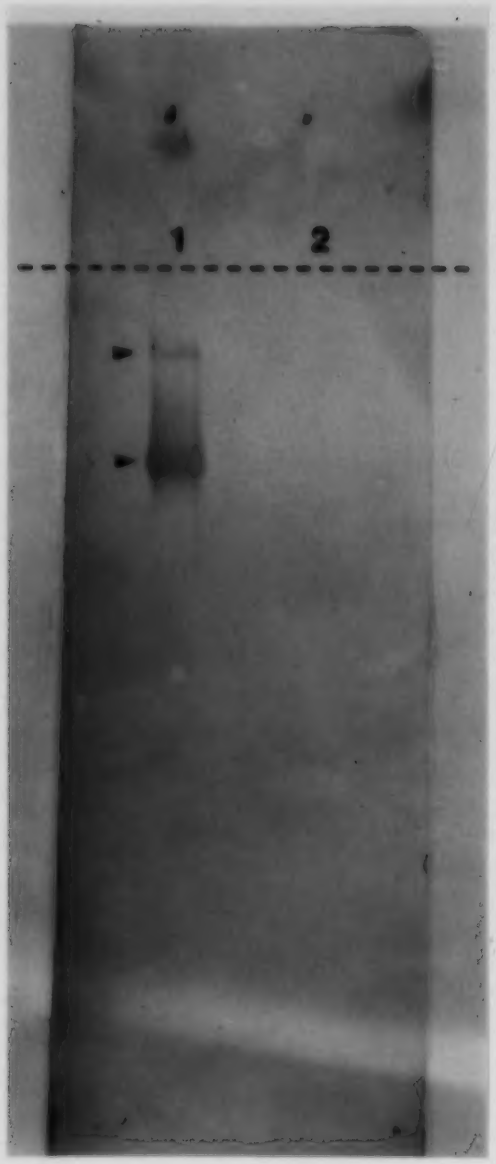
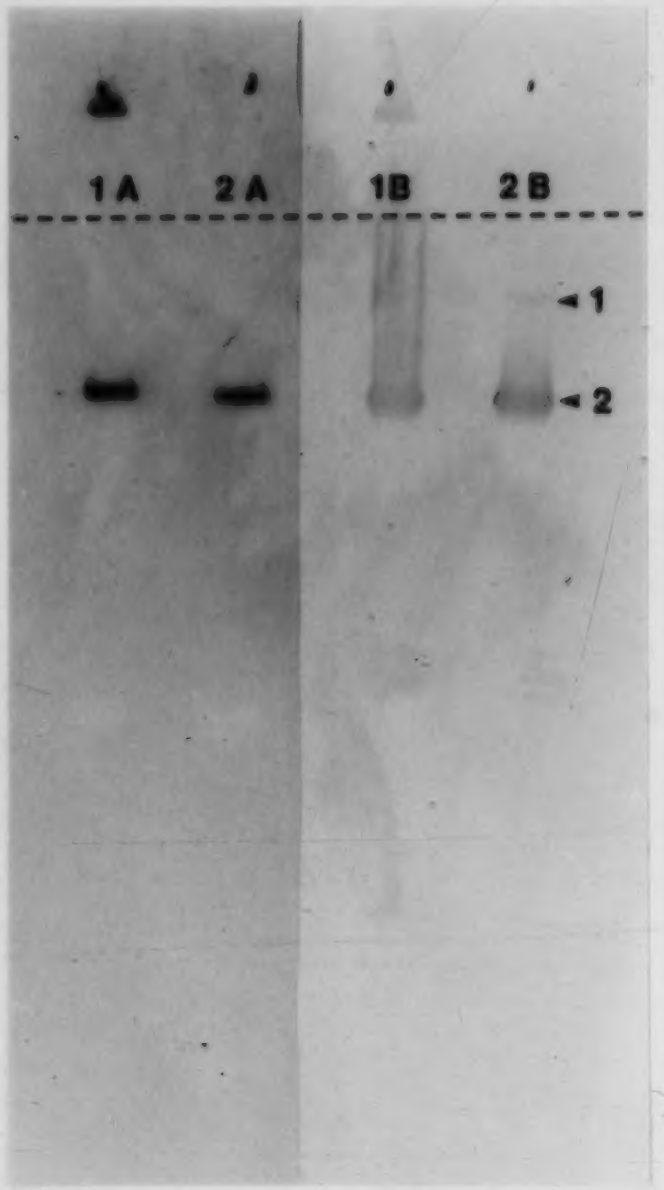

1
2

Fig. 15. Comparison of the Fishbein stain and the Immunoblot to Detect Urease. Shown is a blot of strain 8 cell lysate after electrophoreses in an 8% non-reducing, non-denaturing polyacrylamide gel. Urease activity in lane 1A and 2A were detected by the Fishbein stain. Lanes 1B and 2B were developed by the immunoblot procedure outlined in the text. The cell lysates in lanes 2A and 2B were centrifuged at 12,800 g for 20 seconds prior to electrophoresis.


1A
2A
1B
2B
1
2

The procedure of Hedrick and Smith (17) was utilized to determine the molecular weight (as described in Chapter II) of the two bands detected by the anti-urease antiserum in strain 8 lysate. From the Perguson plot (fig. 16), the retardation coefficient for the band with urease activity (herein termed band 2) was found to be -0.211 and, for the band without detectable urease activity (herein termed band 1) the retardation coefficient was -0.122. The approximate molecular weight was determined to be 740,000 for band 1 and 400,000 for band 2.

The presence and relative mobility of the two bands detected by the antiserum were unchanged (fig. 17) regardless if the cell lysate was treated with either DTT (lane 5) or DTT and iodoacetamide (lane 6) as described in Chapter II.

Immunoblot of SDS-Gels

The immunoblot of strain 8 cell lysate, after electrophoresis in an SDS-polyacrylamide gel (described in Chapter II), revealed two bands (fig. 18) with approximate molecular weights of 179,000 and 174,000. After treatment of the cell lysate with mercaptoethanol and SDS, only the 179,000 dalton band was detected by the antiserum.

Cross Reactivity

After electrophoresis of ureaplasma strains 1, 4, 5, 6 and 8 in an 8% polyacrylamide gel, the protein was

Fig. 16. Ferguson Plot of the Two Bands Detected in Strain 8 Cell Lysate. A plot of Log(Rf X 100) versus acrylamide concentration for the two bands detected in the lysate of strain 8 by the anti-urease antiserum. The retardation coefficient of the band without detectable urease activity (band 1) was found to be -0.122 while the band (band 2) that corresponds to active urease had a coefficient of -0.211 The retardation coefficients were determined by linear regression.

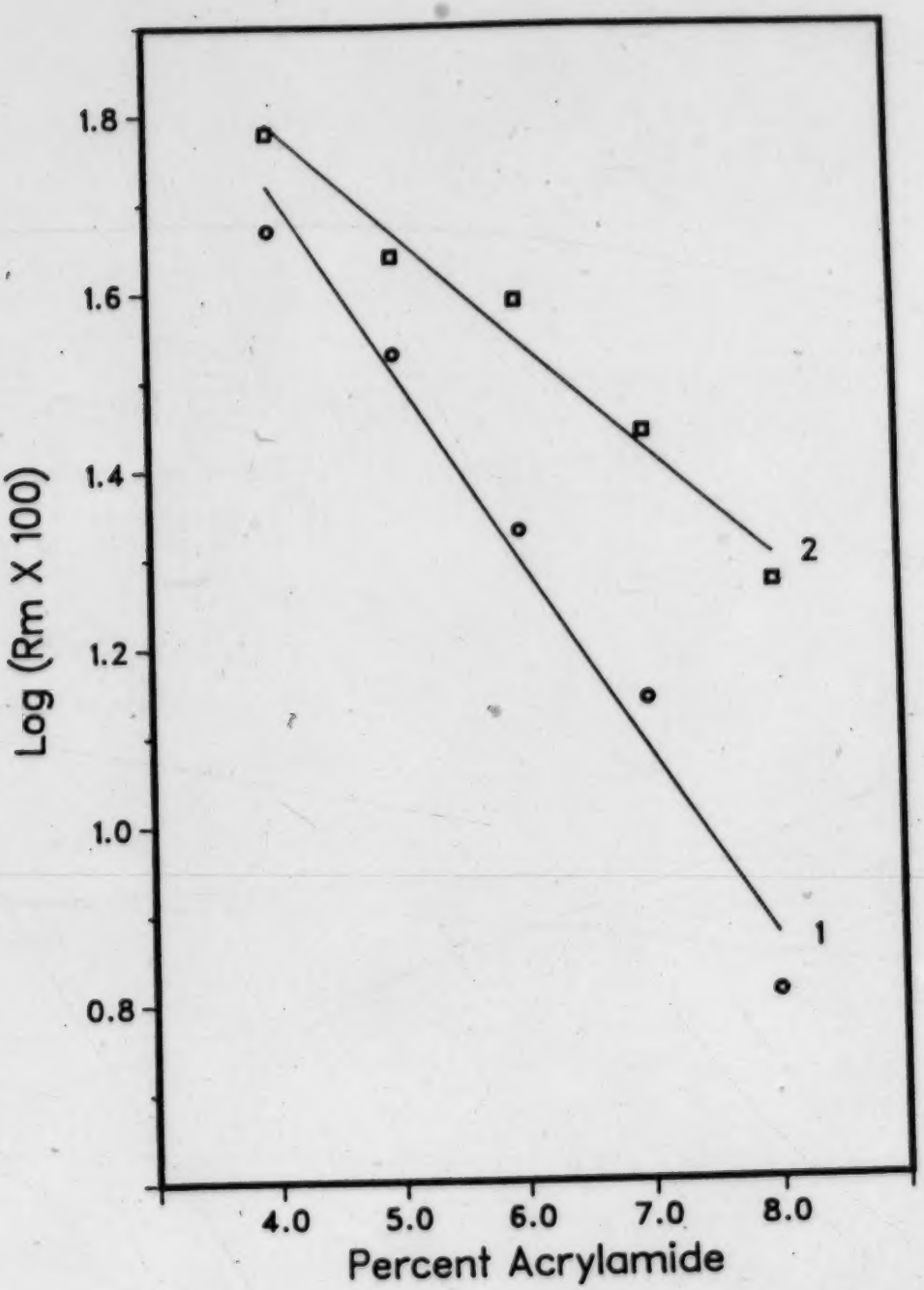
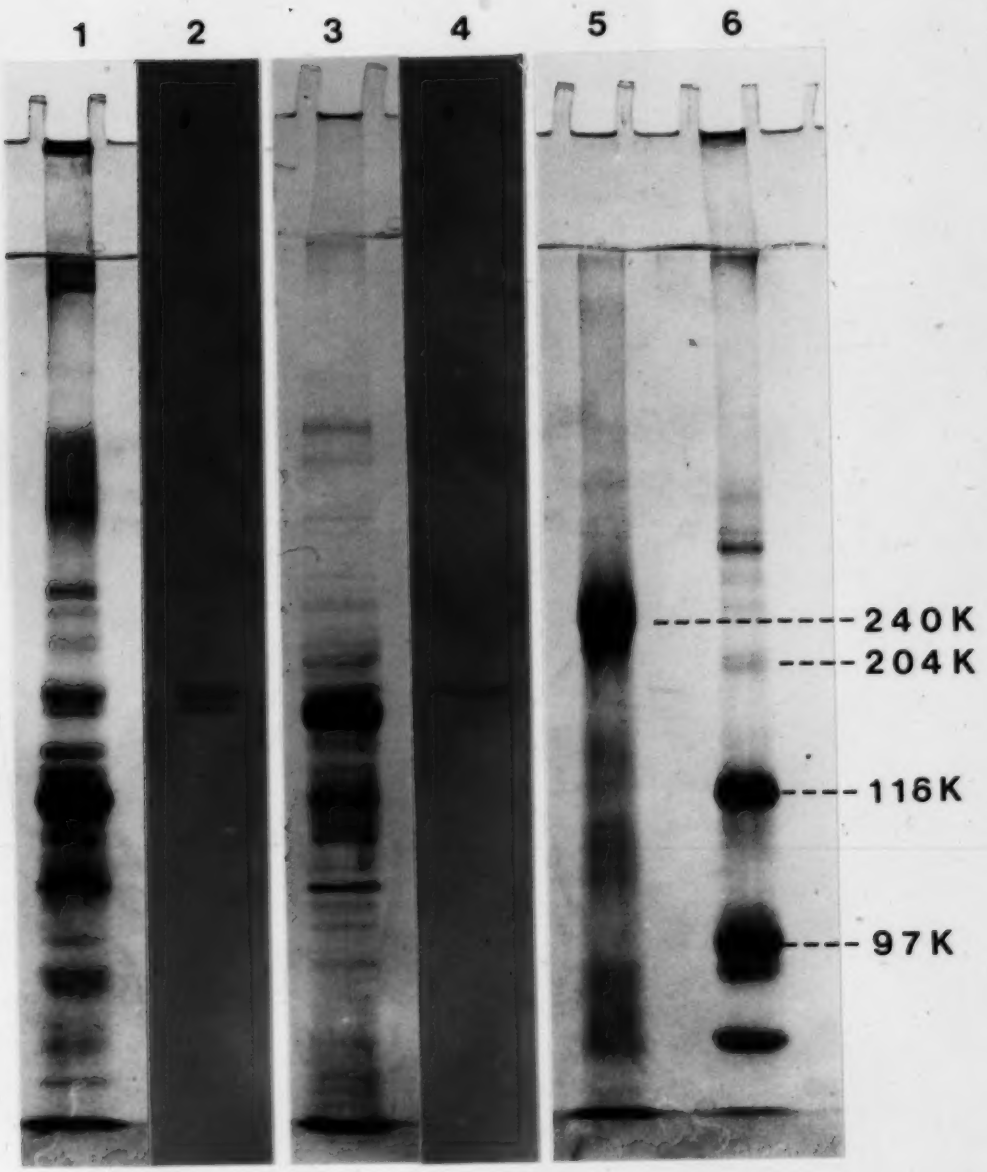

1.8
1.6
1.4
1.2
1.0
0.8
Log (Rm X 100)
2
1
4.0
5.0
6.0
7.0
8.0
Percent Acrylamide

Fig. 17. Immunoblot to Determine the Effects of DTT on Ureaplasma Urease. Shown is a blot of strain 8 lysate subjected to electrophoresis in a 8% non-denaturing, non-reducing polyacrylamide gel. Lanes 1 to 3 show urease activity as visualized by the Fishbein stain. Lanes 4 to 6 show bands detected by the anti-urease antiserum. Both lanes 1 and 4 were untreated, lanes 2 and 5 were treated with DTT for 2 hours, and lanes 3 and 6 were treated with DTT and iodoacetamide as described in the text.

1
2
3
4
5
6

Fig. 18. Immunoblot of Strain 8 Cell Lysate after Electrophoreses in an SDS Gel. Lanes 2 and 4 show a blot of the cell lysate after electrophoresis in an 8% SDS polyacrylamide gel. Lanes 1, 3, 5 and 6 are silver stained portions of the same SDS gel. Prior to electrophoresis, the cell lysate in lanes 1 and 2 were subjected to denaturing conditions only, while in lanes 3 and 4 both denaturing and reducing condition were used. Lanes 5 and 6 contain the molecular weight markers: jack bean urease monomer (240,000), myosin (204,000), phosphorylase (116,000), and β-galactosidase (97,000).


1
2
3
4
5
6
240K
204K
116K
97K

electroblotted to nitrocellulose, which was later developed by the immunoblot procedure. Band 2 was present in all the strains (fig. 19), while band 1 was evident only in strains 5, 6, and 8. In strains 4 and 5 a third band of uncertain identity was observed migrating ahead of band 2.

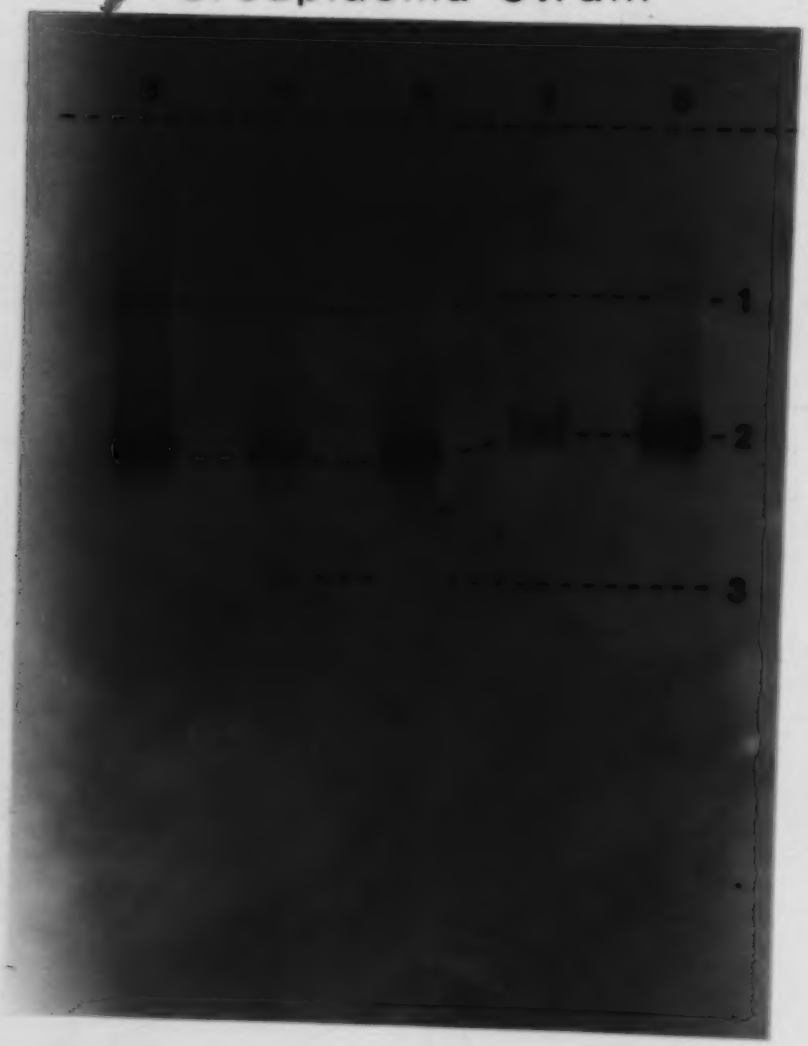
D. Discussion

None of the ureaplasma strains had their urease activity completely neutralized by the anti-urease antiserum. However at high concentrations of antiserum, a constant residual activity was observed for strains 8, 3, and the bovine isolate. Such residual activity is characteristic of antibody-enzyme interactions (40, 10), and may indicate that: 1) all enzyme molecules are partially neutralized equally (that is, all antibodies have the same neutralizing capacity), or 2) the antibody population is heterogenous with regards to neutralizing capacity. The latter probably explains the lack of complete neutralization, because the antiserum was not monoclonal, and because numerous epitopes (that will elicit a heterogenous response) are expected on macromolecules such as ureaplasma urease. Such heterogenous antibody reactions are characteristic of many antibody-enzyme interactions (5, 37, 28).

I expected that neutralization by the anti-urease antiserum would be proportional to the relatedness between the urease of the test strain and strain 8. Based on this, strain 8 urease should exhibit the greatest neutralization

Fig. 19. Cross Reactions of Ureaplasma Urease using anti-Urease Antiserum. Shown is an immunoblot of strains 8, 4, 5, 1 and 6 in lanes 1 to 5 respectively. A major band (2) is evident in all strains, corresponding to ureaplasma urease (hence the difference in migration of band 2 in strains 1 and 6 as demonstrated by the molecular weight determination data). Two other bands designated "1", seen in strains 8, 5, and 6, and "3", seen in strains 4 and 5, are also visible.

Ureaplasma Strain



and not the least as observed (17%). A hypothesis to explain the above results will be presented.

Cinader and Lafferty (12) studying ribonuclease and Ng and Gregory (28) studying lactate dehydrogenase have shown antisera against these enzymes possess two sets of antibodies, one that combines and inhibits enzyme activity and another that combines but does not inhibit. Furthermore, these two sets of antibodies competitively bind to the enzyme such that once one type of antibody has bound the other can not. Knowing this, and that the antiserum is polyclonal, it is possible that the anti-urease antiserum possesses both inhibiting antibodies (I-antibodies), and antibodies that prevent neutralization (P-antibodies). It follows then, that urease must have two epitopes designated I and P.

A final assumption is that the concentration of anti-P antibodies is greater than that of the anti-I antibodies. From this, the low level of neutralization observed when anti-urease antiserum is combined with strain 8 urease may be explained by the predominance of bound anti-P antibodies. However, when heterologous urease is reacted the physical similarity, or cross reactivity, of the P and I epitopes will dictate the extent of neutralization. For example, if the P epitope of strain 3 urease is different from strain 8, but the I epitope is similar, the overall effect will be neutralization due to bound anti-I antibodies. Using this model, there are four possible outcomes of enzyme-antibody

interaction:

1) low cross reactivity of both I and P epitopes to homologous epitopes, resulting in minor neutralization.

2) low cross reactivity of the I epitope but high cross reactivity of the P epitope, yeilding normal activity.

3) high cross reactivity of the I epitope but low cross reactivity of the P epitope resulting in low enzyme activity.

4) high cross reactivity of the I and P epitopes resulting in near normal activity due to the predominance of the anti-P antibodies.

As urease from all ureaplasma strains examined were neutralized by the anti-urease antiserum one can speculate that the I epitope must be conserved. Variation in neutralization levels of heterologous urease suggests either the P epitope differs with each strain, or that the I epitope varies with each strain and the P epitope is absent in heterologous urease.

Based on the extent of neutralization (Table 3), ureaplasma strains may be arbitrarily divided into three groups. The first consists of strains 1 and 8, the second of strains 2, 3, 4, and 5, and the third of the avian, bovine and simian strains. The bovine strain urease revealed high susceptibility to antibody neutralization throughout the antiserum dilution range of 1:100 to 1:500,000 (fig. 11). Hence, the neutralization value was not, in this case, a reliable indicator, and the bovine strain was grouped with

the avian and simian strains.

As the bovine isolates have been shown to be different from the human isolates (based on polypeptide fingerprints and DNA hybridization studies (19)), it is possible, but not necessary, that urease from animal and human isolates are different. If only the I epitope determined the extent of neutralization (that is, the P epitope is only present in strain 8) then urease from animal isolates should be neutralized less than urease from human isolates due to the phylogenetic distance. However, as urease from animal strains were neutralized more than human isolates, the extent of neutralization must be determined by the P epitope. The P epitope of the animal isolates must be different from the human isolates, thereby permitting the binding of anti-I antibodies.

If the extent of cross neutralization is used to judge phylogenetic relatedness, as was done with cytochrome c (24) and trypsin (3), then urease of the avian, bovine and simian strains must have diverged early on from the other strains, while strains 1 and 8 diverged from 2, 3, 4, and 5 later on. As the cluster analysis was based on the homology of a few epitopes on a single protein, it is unlikely that this analysis can be compared to the grouping of the human isolates established by genetic and peptide analysis (9, 20) which grouped strains 1, 3, and 6 together, and 2, 4, 5, and 8 together.

If the model is correct, then absence of neutralization for urease from jack bean and the bacteria examined in this study suggests they lack the I epitope. It can be argued that the urease from these sources have a P epitope identical to that of ureaplasma strain 8, but this is unlikely as strain 8 urease was neutralized 17% by its own antiserum. Although it has been suggested that some genera of the *Mycoplasmataceae* arose by degenerative evolution (27, 23, 42), no link was found between ureaplasma urease, and the few bacterial ureases examined, based on cross neutralization. Presently, there is insufficient data to determine whether the two serologically different forms of urease arose by convergent or divergent evolution.

In summary, we have suggested a model in which the anti-urease antiserum possesses two types of antibodies, one that neutralizes (I-antibodies), and another that prevents neutralization (P-antibodies) without significantly altering enzymatic activity. As anti-I antibodies can either induce a conformational change, or bind directly to the catalytic site, the location of the I epitope is uncertain. Anti-P antibodies may sterically hinder binding of anti-I antibodies to the I epitope, indicating I and P epitopes are in close proximity; alternatively, the two epitopes may be distant, but anti-P antibodies induce a conformational change in the enzyme that prevents the binding of anti-I antibodies. The I epitope appears to be highly conserved throughout the genus, while the proposed P epitope is

variable and determines the extent of neutralization induced by the anti-urease antiserum.

To test this model, one could fractionate the anti-urease antiserum (12, 28) and check for protecting and/or neutralizing antibodies. Several studies (37, 29, 10) have isolated activating antibodies in sera judged as having an overall neutralizing affect. Presence of activating antibodies in the anti-urease antisera is unknown and again can only be determined by fractionation. Alternatively, one could obtain anti-urease antisera against another strain and re-examine the neutralization pattern. If the proposed model is correct, then the homologous strain urease should be neutralized less than heterologous urease. If the P epitope is characteristic only of strain 8, then the homologous strain urease should exhibit the most neutralization and the heterologous the least.

The antiserum was shown to react with two prominent components in the lysate of strain 8, designated bands 1 and 2. The antiserum was judged to be monospecific by immunodiffusion analysis using whole ureaplasma cell lysates. However, when using a more sensitive assay requiring simple binding of antibody to antigen, such as the immunoblot, it is possible that antigens other than urease may also react. Thus the other faint bands observed may represent either degradation or aggregation products of the prominant bands, or proteins that cross react with strain 8 urease. Band 2 corresponded to ureaplasma urease and had an approximate

molecular weight of 400,000. Band 1 had an approximate molecular weight of 740,000, but was not detected by the Fishbein stain. As multiple forms of jack bean urease are known to result from polymerization of a single subunit (16), and since Romano et al. (32) reported two bands of active urease with similar relative mobility as those observed in the immunoblot, it is possible that band 1 may be an aggregated form of ureaplasma urease with undetectable enzymatic activity. Romano et al. (32) then, may not be observing isoenzymes (by the definition adopted in this thesis) but urease aggregates. The activity reported by Romano et al. (32) in the higher molecular weight forms may have been due to the acetone purification procedure (which might promote aggregate formation), or they may have subjected more cell material to electrophoresis.

The immunoblot of the SDS gel revealed 2 bands calculated to be 179,000 and 174,000 daltons which might indicate that ureaplasma urease is a heterodimer. The 174,000 unit might be composed of further subunits joined by disulfide bonds, but which are not immunologically reactive. Subunits of urease were not detected by the anti-urease antiserum in the lysate of strain 8 after native gel electrophoresis. However a band (herein termed band 3) at an Rf of 0.56 in the lysates of strains 4 and 5 was detected by the antiserum (fig. 19) that lacked enzymatic activity. Although the molecular weight of band 3 was not determined experimentally, it can be shown (Appendix B) that the

relative mobility of band 3 is similar to a hypothetical 200,000 urease subunit (Rf = 0.58). Why band 3 was detected only in the lysates of strains 4 and 5 is unknown. As the cell lysates of strains 4 and 5 were several months old, thermal instability (26) leading to denaturation coupled with possible proteolytic digestion may have contributed to the dissociation of the monomeric form into its subunit.

After reduction and alkylation there was loss of enzymatic activity, though no detectable molecular dissociation was observed. If ureaplasma urease possesses numerous disulfide bonds close to the surface of the molecule similar to jack bean urease (18, 30), then introduction of alkyl groups may cause a conformational change and subsequent loss of activity. Equally possible is that disulfide bonds exist close to the catalytic site which, after reduction and alkylation, result in acetamide groups projecting into the catalytic site which reduce activity by steric hinderance.

The antiserum was shown to react with ureaplasma urease and at least two other components of the cell, believed to be molecular weight variants of "monomeric" urease. Further confirmation of these results could be obtained if purified urease, or antiserum specific for urease, were available. Of interest would be to determine the exact subunit composition and the possibility of various aggregation states. With purified urease the number of accessible disulfide bonds may be determined by titration, and may offer an explanation of the effects of reduction and alkylation.

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IV. General Discussion and Conclusions

We have examined some of the kinetic data pertaining to ureaplasma urease using the rapid and convenient Berthelot assay. The pH optimum of four strains was found to be approximately 7.2, and the Km was approximately 2.5 mM urea. The molecular weight of ureaplasma urease is approximately 390,000 based on native gel electrophoresis, and immunoblots (using rabbit anti-urease antiserum). From immunoblots and SDS gel electrophoresis we hypothesized the 390,000 unit to consist of two monomers of approximately 150 - 170,000 daltons, with one of the monomers being composed of further protomers. As well, the 390,000 unit may aggregate to form a dimer of approximately 740,000 daltons.

Growth at an increased concentration of urea had no effect in inducing formation of other enzymatic forms that might have a pH optimum different from 7.2, or that possessed a different electrophoretic mobility upon native gel electrophoresis. Multiple enzyme forms were only detected by isoelectrofocusing the cell lysate. Most of the ureaplasma strains examined possessed 2 to 3 enzymatic forms with pI values equal or close to 5.2.

From neutralization data using rabbit anti-urease antiserum, we proposed a model in which ureaplasma urease possessed two epitopes, I (inhibiting) which was invariant and responsible for neutralization and P (protecting) which was variable and determined the extent of neutralization, possibly by preventing the combination of I-antibodies with

the I epitope. The ureaplasma strains examined could be divided into three groups based on the extent of neutralization. Urease of the animal strains (ovine, bovine, and simian) are distinct from that of the human isolates, further supporting studies that suggest human and animal isolates are different.

Attempts to purify urease were unsuccessful, making complete characterization of ureaplasma urease and further confirmation of the data presented here not yet possible. Although there are a number of prospective methods to purify urease, two of particular interest would be generation of monoclonal antibodies for the synthesis of a specific immunoaffinity column, and use of cloning techniques for production of pure enzyme. As these are established techniques, pure urease may be obtained in the foreseeable future.

The interest in *Ureaplasma urealyticum* results from both its medical significance in sexually transmitted diseases, and its biological significance as being one of the smallest free living organisms. Ureaplasma urease is of interest because it is essential to survival of the organism, its biological role is unknown, and it may be useful as a target for chemotherapeutic agents in the control of ureaplasma infections. This study was undertaken to further elucidate the biochemical properties, both kinetic and physical, of ureaplasma urease. Though we are slowly gaining a better picture of the biochemical and biological

properties of ureaplasma urease, many questions remain unanswered. Perhaps these answers will be evident when we overcome the hurdle of purifying and characterizing the enzyme. Any gains in knowledge of this essential enzyme may have consequences in both the medical and biological worlds, with all gains contributing to the understanding of the organism as a whole.

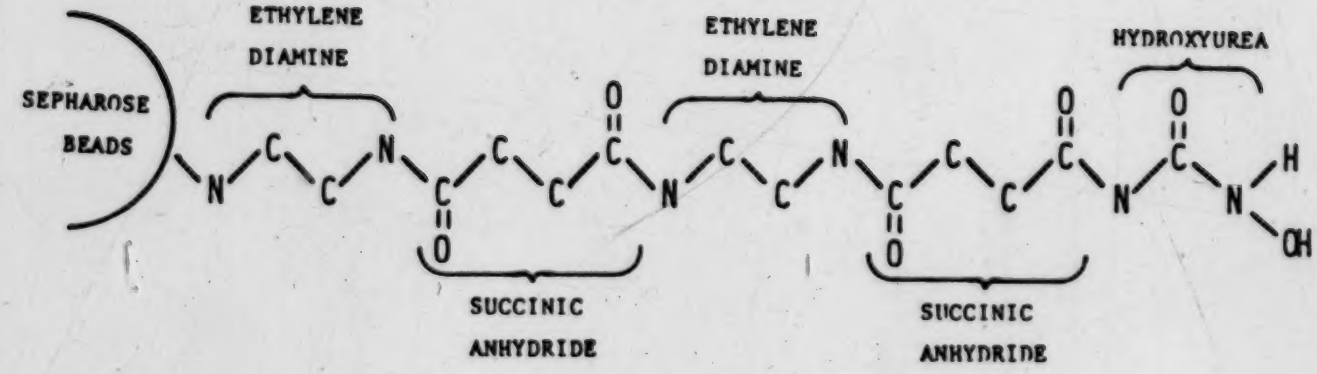
V. Appendices

Appendix A.
Hydroxyurea Affinity Column

A Sepharose 4-B hydroxyurea affinity column was prepared using the procedure of Wong and Shobe (3), and of Cuatrecasas (1). A long chain linker arm attached to Sepharose beads was synthesized by repeated addition of ethylene diamine and succinic anhydride. Hydroxyurea was then coupled to the free end of the linker arm to form the complete affinity column (fig. 20). Sepharose 4-B was purchased from Pharmacia, and cyanogen bromide (CNBr) from Eastman Kodak. Succinic anhydride, hydroxyurea, ethylene diamine, and water soluble carbodiimide (N-ethyl-N'-[3-dimethyl aminopropyl] carbodiimide) were purchased from Sigma.

To activate the Sepharose, CNBr (150 mg/ml of packed beads) was added. The pH of the solution was immediately raised to, and maintained at, 11 by addition of 2 N NaOH. Once the pH of the slurry no longer dropped, the beads were transferred to a sintered glass funnel and washed with a large volume of 0.1 M borate saline buffer (pH 8.0). The pH of the activated beads in borate saline buffer was then raised to 10. An equal volume of ethylene diamine (2 mmol/ml of packed beads) dissolved in distilled water was added and the pH maintained at 6 by addition of 6 N HCl. The reaction continued overnight at 4°C before washing the aminoethyl-Sepharose beads with large volumes of distilled water and borate saline buffer. Succinic anhydride

Fig. 20. Hydroxyurea Affinity Column. Theoretical representation of the hydroxyurea affinity column, with two repeating units of ethylene diamine and succinic anhydride cross linking hydroxyurea to the Sepharose bead.


SEPHAROSE
BEADS
ETHYLENE
DIAMINE
SUCCINIC
ANHYDRIDE
ETHYLENE
DIAMINE
SUCCINIC
ANHYDRIDE
HYDROXYUREA

(1 mmol/ml of packed beads) was dissolved in distilled water. The pH of the aminoethyl-Sepharose was lowered to 6 before adding the succinic anhydride, and then maintained at pH 6 by the addition of 5 M NaOH. After the pH stabilized, the reaction was allowed to continue for an additional 5 hours at 4°C. The succinylaminoethyl-Sepharose was then washed with water and borate saline buffer as before.

To couple an additional ethylene diamine to the succinylaminoethyl-Sepharose, the pH of the beads was lowered to 5.0 with 6 M HCl. Ethylene diamine (2 mmol/ml of packed beads) was added, followed by dropwise addition of 500 mg of carbodiimide dissolved in 3 ml of water. The pH was maintained at 5 by adding 2 N NaOH, and the reaction was allowed to continue for 20 hours at room temperature before extensively washing the beads with water and borate saline buffer. To complete the synthesis of the long chain linker arm, another succinic anhydride moiety was attached to the beads by repeating the procedure of coupling succinic anhydride to ethylene diamine.

The final step involved coupling hydroxyurea to the free carboxyl terminus of the linker arm. The pH of the beads was lowered to 6.0, and hydroxyurea (4.36 mg/ml of packed beads) was dissolved in a minimum volume of water, and added with stirring. Carbodiimide (26.4 mg/ml of packed beads) was dissolved in a minimum volume of water and added dropwise to the slurry. The reaction continued for 20 hours at 4°C before washing with water and borate saline buffer.

The completed hydroxyurea coupled Sepharose beads were equilibrated with phosphate buffer pH 7.0 and packed into a 10 ml syringe.

Appendix B
Determination of the Rf Value for a Hypothetical 270,000 Subunit of Urease

The 2 bands detected in the lysate of strain 8 have a molecular weight of 400,000 (band 2) and 740,000 (band 1). If band 1 is a polymer of band 2, then their corresponding lines from the Ferguson plot should intersect (2). To determine the coordinates of the intersection point, the y-value (or Log(Rf X 100) value) of the slope equation for each line was set to equal one another:

$$m_1x_1 + b_1 = y_1 = y_2 = m_2x_2 + b_2$$

where m, x, and b are the slope, x-axis value (or acrylamide concentration), and the y-axis intercept respectively for bands 1 and 2. This equation may be rearranged to:

$$m_1x_1 - m_2x_2 = b_2 - b_1 \quad (1)$$

From the data in Chapter III, band 1 has a slope (m_1) of -0.211, and band 2 a slope (m_2) of -0.122 By linear regression, the y-axis intercept for bands 1 and 2 were determined to be 2.56 (b_1) and 2.28 (b_2). Substituting these values into equation (1):

$$-0.211x + 0.122x = 2.28 - 2.56$$

$$-0.089x = -0.28$$

$$x = 3.15$$

at x = 3.15 the corresponding y value is:

$$y = mx + b$$

$$y = -0.211x + 2.56$$

$$y = -0.211(3.15) + 2.56$$

$$y = 1.89$$

Therefore the point (3.15, 1.89) is where the two lines intersect (fig. 21).

If ureaplasma urease is composed of subunits with a molecular weight of approximately 200,000, then the retardation coefficient, or slope (m_3), for this subunit would be -0.070 (fig. 22). Knowing that the subunit must also be described by a line that passes through the point (3.15, 18.9), the relative mobility of the subunit at any given acrylamide concentration may by found by the following equation:

$$y_1 - y_2 = m_3(x_1 - x_2)$$

$$y_1 - 1.89 = -0.070 \ (\ x_1 - 3.15 \ )$$

At a 5% polyacrylamide concentration the subunit would have an Rf of:

$$y_1 = -0.070(\ 5 - 3.15 \ ) + 1.89$$

$$y_1 = \text{Log}(Rf \ X \ 100) = 1.77$$

$$Rf \ X \ 100 = 57.7$$

$$Rf = 0.58$$

Hence the Rf value for a 200,000 subunit of urease would be 0.58 in a 5% polyacrylamide gel.

Fig. 21. Ferguson Plot for a Hypothetical Ureaplasma Urease Subunit. A Ferguson plot showing the data obtained for the two bands detected by the anti-ureaplasma urease antiserum in the lysate of strain 8 (designated 1 and 2). These lines were extrapolated to a common point (3.15, 1.89). Assuming a subunit of ureaplasma urease has a molecular weight of 200,000 daltons, and knowing that it must pass through the point (3.15 1.89), a line that describes the mobility of the subunit as a function of changing acrylamide concentration can be drawn (dashed line designated 3). The point (5, 1.75) (Δ) corresponds to the position of the actual band (band 3) detected in the lysates of strains 4 and 5 by the anti-urease antiserum.

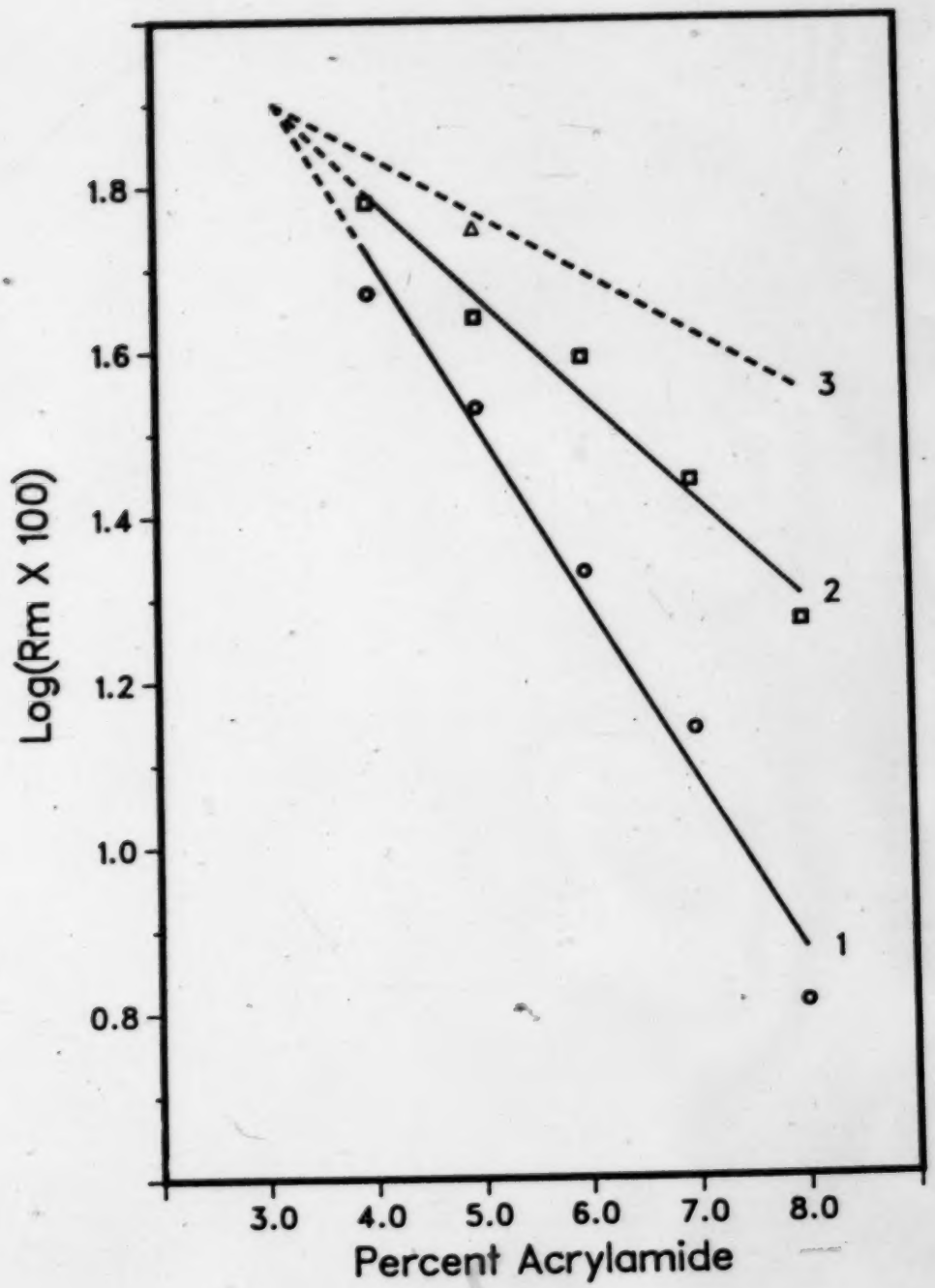
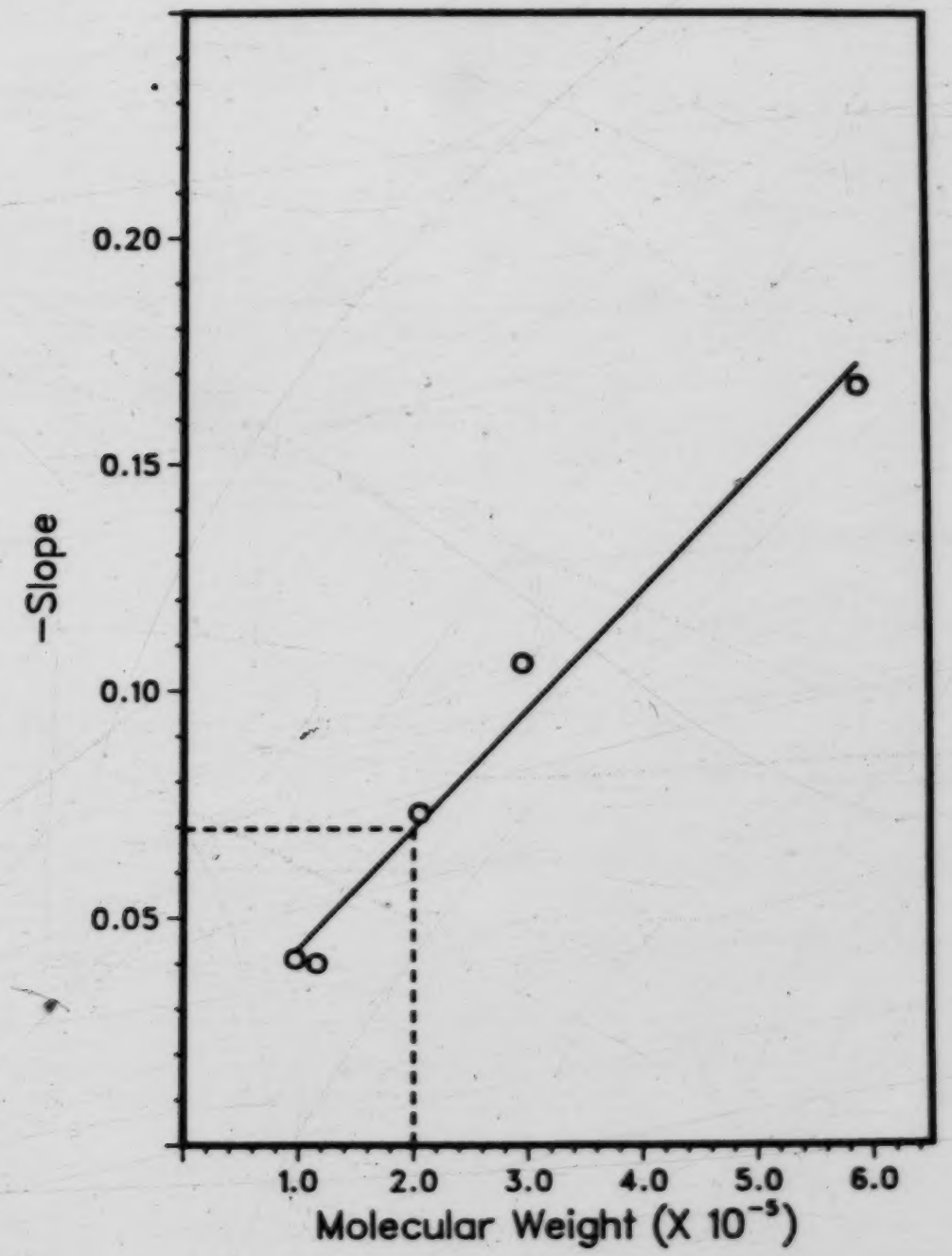

Log(Rm X 100)
1.8
1.6
1.4
1.2
1.0
0.8
3.0
4.0
5.0
6.0
7.0
8.0
Percent Acrylamide
1
2
3

Fig. 22. Retardation Coefficient for a Hypothetical 200,000 Dalton Subunit of Ureaplasma Urease. From the standard curve of retardation versus molecular weight (Chapter II), a ureaplasma urease subunit of 200,000 daltons would have a retardation coefficient of -0.070 (dashed lines).


0.20
0.15
0.10
0.05
−Slope
1.0
2.0
3.0
4.0
5.0
6.0
Molecular Weight (X 10⁻⁵)

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